

430 Rec'd PCT/PTO 09/24/99 29 SEP

U.S. DEPARTMENT OF  
Patent and Trade  
Docket No. 22TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. § 371

U.S. APPLICATION NO. (If known, see 37 C.F.R. § 1.5)

INTERNATIONAL APPLICATION NO.  
PCT/US98/23496INTERNATIONAL FILING DATE  
4 November 1998PRIORITY DATE CLAIMED  
10 November 1997TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR IDENTIFYING VARIATIONS IN HUMAN  $\alpha_{1B}$ - AND  $\beta_2$ -  
ADRENERGIC RECEPTOR GENES

APPLICANT(S) FOR DO/EO/US: Paul A. Insel, Volker Herrmann, Rainer Buescher and Stephen B. Liggett

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. § 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. § 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. § 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. § 371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ has been transmitted by the International Bureau.
  - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. § 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. § 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. § 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. § 371(c)(5)).

**Items 11. to 16. below concern document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. §§ 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.  
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and of address.
16. ☒ Other items or information: return receipt postcard.


**CERTIFICATE OF MAILING BY "EXPRESS MAIL"**

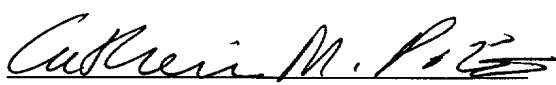
Express Mail Label No : EL 034 669 837 US

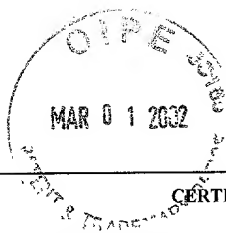
Date of Deposit: September 29, 1999

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

  
Gary Paulazzo

U.S. APPLICATION NO. (If known, see 37 C.F.R. § 1.5) <b>02/100244</b>		INTERNATIONAL APPLICATION NO. PCT/US98/23496		DOCKET NUMBER: 220002058901	
17. <input checked="" type="checkbox"/> The following fees are submitted: <b>BASIC NATIONAL FEE (37 C.F.R. §§ 1.492(a)(1)-(5)):</b> Search Report has been prepared by the EPO or JPO ..... \$840.00 International preliminary examination fee paid to (USPTO (37 C.F.R. § 1.482)) ..... \$670.00 No international preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) but international search fee paid to USPTO (37 C.F.R. § 1.445(a)(2)) ..... \$760.00 Neither international preliminary examination fee (37 C.F.R. § 1.482) nor international search fee (37 C.F.R. § 1.445(a)(2)) paid to USPTO ..... \$970.00 International preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... \$96.00				<b>CALCULATIONS PTO USE ONLY</b>	
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				\$670.00	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(e)).				\$0.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	36 - 20 =	16	x \$18.00	\$288.00	
Independent claims	2 - 3 =	0	x \$78.00	\$0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$0.00	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$958.00	
Reduction by ½ for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 C.F.R. §§ 1.9, 1.27, 1.28)				\$479.00	
<b>SUBTOTAL =</b>				\$479.00	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(f)).				+	\$0.00
<b>TOTAL NATIONAL FEE =</b>				\$479.00	
Fee for recording the enclosed assignment (37 C.F.R. § 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§ 3.28, 3.31). <b>\$40.00 per property</b>				+	\$0.00
<b>TOTAL FEES ENCLOSED =</b>				\$479.00	
				Amount to be refunded:	\$
				charged:	\$
a. <input type="checkbox"/> A check in the amount of \$ _____ to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my <b>Deposit Account No. 03-1952</b> in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Assistant Commissioner is hereby authorized to charge any additional fees that may be required, or credit any overpayment to <b>Deposit Account No. 03-1952</b> .					
<b>NOTE: Where an appropriate time limit under 37 C.F.R. § 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. § 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>					
SEND ALL CORRESPONDENCE TO: Catherine M. Polizzi Morrison & Foerster LLP 755 Page Mill Road Palo Alto, California 94304-1018					
 SIGNATURE Catherine M. Polizzi Registration No. 40,130					

U.S. APPLICATION NO. (if known, see 37 C.F.R. § 1.53) <b>09/402274</b>		INTERNATIONAL APPLICATION NO. PCT/US98/23496		DOCKET NUMBER: 220002058	
17. <input checked="" type="checkbox"/> The following fees are submitted: <b>BASIC NATIONAL FEE (37 C.F.R. §§ 1.492(a)(1)-(5)):</b> Search Report has been prepared by the EPO or JPO ..... \$840.00 International preliminary examination fee paid to (USPTO (37 C.F.R. § 1.482))..... \$670.00 No international preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) but international search fee paid to USPTO (37 C.F.R. § 1.445(a)(2)) ..... \$760.00 Neither international preliminary examination fee (37 C.F.R. § 1.482) nor international search fee (37 C.F.R. § 1.445(a)(2)) paid to USPTO ..... \$970.00 International preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)..... \$96.00				<b>CALCULATIONS PTO USE ONLY</b>	
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$670.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(e)).				\$0.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	36 - 20 =	16	x \$18.00	\$288.00	
Independent claims	2 - 3 =	0	x \$78.00	\$0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$958.00	
Reduction by ½ for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 C.F.R. §§ 1.9, 1.27, 1.28)				\$479.00	
SUBTOTAL =				\$479.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(f)).				+	\$0.00
TOTAL NATIONAL FEE =				\$479.00	
Fee for recording the enclosed assignment (37 C.F.R. § 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§ 3.28, 3.31). \$40.00 per property				+	\$0.00
TOTAL FEES ENCLOSED =				\$479.00	
				Amount to be refunded:	\$
				charged:	\$
a. <input type="checkbox"/> A check in the amount of \$ _____ to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my <u>Deposit Account No. 03-1952</u> in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Assistant Commissioner is hereby authorized to charge any additional fees that may be required, or credit any overpayment to <u>Deposit Account No. 03-1952</u> .					
<b>NOTE: Where an appropriate time limit under 37 C.F.R. § 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. § 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>					
SEND ALL CORRESPONDENCE TO:  Catherine M. Polizzi Morrison & Foerster LLP 755 Page Mill Road Palo Alto, California 94304-1018					
 SIGNATURE					
Catherine M. Polizzi <u>Registration No. 40,130</u>					



PATENT  
Docket No. 220002058901  
Client Ref. UC 98-021-3

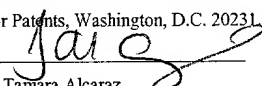
#  
13

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

Express Mail Label No.: EL779890398US

Date of Deposit: March 1, 2002

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

  
Tamara Alcaraz

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Paul A. INSEL et al.

Serial No.: 09/402,244

371 Filing Date: September 29, 1999

For: METHODS AND COMPOSITIONS FOR  
IDENTIFYING VARIATIONS IN  
HUMAN  $\alpha_{1B}$ - AND  $\beta_2$ - ADRENERGIC  
RECEPTOR GENES

Examiner: To Be Assigned

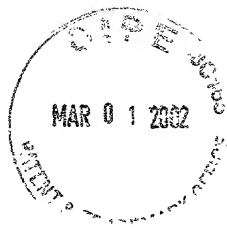
Group Art Unit: 5611

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

Prior to examination on the merits, Applicants respectfully request entry of this  
Preliminary Amendment for the above-identified patent application.



## AMENDMENTS

### In the specification:

Please amend the paragraph on page 10, containing line 1, as follows:

(Amended)

Figure 5 (SEQ ID NO:11) depicts the sequence profile of fragment A of the human  $\alpha_1$ B-AR gene. The amplified fragments were sequenced with the amplification primer SEQ ID NO: 1 using Applied Biosystems' DNA sequencer. Each peak indicates a different nucleotide. In some cases "ambiguous reads" (N) occur as the result of duplicate bases (heterozygosity) or reading ambiguities, which can generally be corrected afterwards.

Please amend the paragraph on page 10, containing line 7, as follows:

(Amended)

Figure 6 (SEQ ID NO:12) depicts the sequence profile of fragment A of the human  $\beta_2$ -AR gene. The amplified fragments were sequenced with the amplification primer SEQ ID NO:6 using Applied Biosystems' DNA sequencer. Each peak indicates a different nucleotide. In some cases "ambiguous reads" (N) occur as the result of duplicate bases (heterozygosity) or reading ambiguities, which can generally be corrected afterwards.

Please amend the paragraph on page 27, containing line 30, as follows:

(Amended)

In a preferred embodiment, the method is used for diagnosing nocturnal asthma based on glycine 16 polymorphism in  $\beta_2$ -AR gene. In another embodiment, the method is used for a diagnosis of essential hypertension based on the same genetic polymorphism. While assaying for glycine 16 polymorphism, genomic DNA of the test subject can be obtained from a blood sample. The N-terminal fragment A (as shown in Figure 2) that encompasses sequences encoding the residue glycine 16 can be amplified using primers SEQ ID NOS:5 and 6, or

derivatives thereof. Direct sequencing of the amplified products using the same primers employed in the amplification procedure may be performed to detect the single base change (adenosine to guanosine) at position 46 (numbering from the start codon) that results in amino acid substitution of arginine with glycine. Homozygosity of glycine 16 indicates the presence or a predisposition to nocturnal asthma and/or essential hypertension. Glycine 16 homozygotes will be apparent by comparing the sequence peaks from the subject sample to those of the controls (for an example of the sequence output from an automated DNA sequencer, see Figure 5 (SEQ ID NO:11) or 6 (SEQ ID NO:12)). A single peak corresponding to 27 nucleotide guanosine at position 46 is indicative of glycine 16 homozygosity. Two overlapping peaks, each representing guanosine or adenosine respectively at position 46, suggest heterozygosity of glycine 16. Finally, a single peak representing adenosine shows that the subject is homozygous in arginine 16.

Please amend the paragraph on page 31, containing line 24, as follows:

(Amended)

#### Example 5

#### Sequence Analysis

The PCR products of expected sizes were cut from the gel and the DNA was purified using QIAquick Gel Extraction Kit. The extracted DNA was resuspended in Tris-EDTA buffer (10mM Tris-Cl, 1mM EDTA, pH 8. 0) and concentrated using a Centricon Concentrator (Amicon). The purified gene fragments were then sequenced by an automated DNA sequencer (Applied Biosystems, model 377) using one or more of the same primers employed in PCR. The upstream primer SEQ ID NO: 1 and the downstream primer SEQ ID NO: 6 were used for sequencing the amplified products, fragment A of the human I<sub>1B</sub>-adrenergic receptor gene and fragment A of 9<sub>2</sub>-adrenergic receptor gene, respectively. As shown in Figure 5 (SEQ ID NO:11) and 6 (SEQ ID NO:12), each sequencing read approximately 550 bases. Other primers described herein including primer SEQ ID NOS: 1, 2, 3, 5, and 7 can also be used for direct sequencing

with high reliability. By use of an automated sequencer and sequencing PCR products from in excess of 15 different subjects, we obtained consistent results in accordance with the published coding sequences of the human  $\beta_2$ - and exon 1 of the human  $I_{1B}$ -adrenergic receptor. Repeated sequencing of PCR products of the same individuals revealed a 100% reliability of our PCR methods without requirement for repeat isolation of PCR fragments. Occasionally occurring "ambiguous reads", which are the result of a reading error of the automated sequencer, can generally be corrected afterwards without re-isolating and sequencing the PCR fragments (Figures 5 and 6).



## REMARKS

In compliance with 37 C.F.R. § 1.821 through § 1.825, applicants have amended the specification to add Sequence Identifiers. No new matter has been added by this amendment. Applicants respectfully request entry of the present amendment.

Attached hereto is a marked up version of the changes made to the specification by the current amendment with additions underlined and deletions bracketed. The attached pages are captioned "VERSION WITH MARKINGS TO SHOW CHANGES MADE".

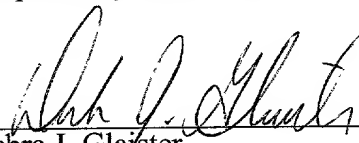
## CONCLUSION

In the unlikely event that the transmittal letter is separated from this document and/or the Patent Office determines that an extension and/or other relief is required, applicants petition for any required relief including extensions of time and authorize the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 220002058901. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

Dated: March 1, 2002

By:

  
Debra J. Glaister  
Registration No. 33,888

for Catherine M. Polizzi  
Registration No. 40,130

Morrison & Foerster <sup>LLP</sup>  
755 Page Mill Road  
Palo Alto, California 94304-1018  
Telephone: (650) 813-5725  
Facsimile: (650) 494-0792



**VERSION WITH MARKINGS TO SHOW CHANGES MADE**

**METHODS AND COMPOSITIONS FOR IDENTIFYING VARIATIONS IN HUMAN  $\alpha_1$ B-  
AND  $\beta_2$ - ADRENERGIC RECEPTOR GENES**

**In the specification:**

The paragraph on page 10, containing line 1, has been amended as follows:

(Amended)

Figure 5 (**SEQ ID NO:11**) depicts the sequence profile of fragment A of the human  $\alpha_1$ B-AR gene. The amplified fragments were sequenced with the amplification, primer SEQ ID NO: 1 using Applied Biosystems' DNA sequencer. Each peak indicates a different nucleotide. In some cases "ambiguous reads" (N) occur as the result of duplicate bases (heterozygosity) or reading ambiguities, which can generally be corrected afterwards.

The paragraph on page 10, containing line 7, has been amended as follows:

(Amended)

Figure 6 (**SEQ ID NO:12**) depicts the sequence profile of fragment A of the human  $\beta_2$ -AR gene. The amplified fragments were sequenced with the amplification primer SEQ ID NO:6 using Applied Biosystems' DNA sequencer. Each peak indicates a different nucleotide. In some cases "ambiguous reads" (N) occur as the result of duplicate bases (heterozygosity) or reading ambiguities, which can generally be corrected afterwards.

The paragraph on page 27, containing line 30, has been amended as follows:

(Amended)

In a preferred embodiment, the method is used for diagnosing nocturnal asthma based on glycine 16 polymorphism in  $\beta_2$ -AR gene. In another embodiment, the method is used for a diagnosis of essential hypertension based on the same genetic polymorphism. While assaying for glycine 16 polymorphism, genomic DNA of the test subject can be obtained from a blood sample. The N-terminal fragment A (as shown in Figure 2) that encompasses sequences

encoding the residue glycine 16 can be amplified using primers SEQ ID NOS:5 and 6, or derivatives thereof. Direct sequencing of the amplified products using the same primers employed in the amplification procedure may be performed to detect the single base change (adenosine to guanosine) at position 46 (numbering from the start codon) that results in amino acid substitution of arginine with glycine. Homozygosity of glycine 16 indicates the presence or a predisposition to nocturnal asthma and/or essential hypertension. Glycine 16 homozygotes will be apparent by comparing the sequence peaks from the subject sample to those of the controls (for an example of the sequence output from an automated DNA sequencer, see Figure 5 (SEQ ID NO:11) or 6 (SEQ ID NO:12)). A single peak corresponding to 27 nucleotide guanosine at position 46 is indicative of glycine 16 homozygosity. Two overlapping peaks, each representing guanosine or adenosine respectively at position 46, suggest heterozygosity of glycine 16. Finally, a single peak representing adenosine shows that the subject is homozygous in arginine 16.

The paragraph on page 31, containing line 24, has been amended as follows:

(Amended)

#### Example 5

#### Sequence Analysis

The PCR products of expected sizes were cut from the gel and the DNA was purified using QIAquick Gel Extraction Kit. The extracted DNA was resuspended in Tris-EDTA buffer (10mM Tris-Cl, 1mM EDTA, pH 8. 0) and concentrated using a Centricon Concentrator (Amicon). The purified gene fragments were then sequenced by an automated DNA sequencer (Applied Biosystems, model 377) using one or more of the same primers employed in PCR. The upstream primer SEQ ID NO: 1 and the downstream primer SEQ ID NO: 6 were used for sequencing the amplified products, fragment A of the human I<sub>1B</sub>-adrenergic receptor gene and fragment A of  $\beta_2$ -adrenergic receptor gene, respectively. As shown in Figure 5 (SEQ ID NO:11) and 6 (SEQ ID NO:12), each sequencing read approximately 550 bases. Other primers

described herein including primer SEQ ID NOS: 1, 2, 3, 5, and 7 can also be used for direct sequencing with high reliability. By use of an automated sequencer and sequencing PCR products from in excess of 15 different subjects, we obtained consistent results in accordance with the published coding sequences of the human  $\beta_2$ - and exon 1 of the human  $I_{1B}$ -adrenergic receptor. Repeated sequencing of PCR products of the same individuals revealed a 100% reliability of our PCR methods without requirement for repeat isolation of PCR fragments. Occasionally occurring "ambiguous reads", which are the result of a reading error of the automated sequencer, can generally be corrected afterwards without re-isolating and sequencing the PCR fragments (Figures 5 and 6).

METHODS AND COMPOSITIONS FOR IDENTIFYING  
VARIATIONS IN HUMAN  $\alpha_{1B}$ - AND  $\beta_2$ -ADRENERGIC RECEPTOR  
GENES

5

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER  
FEDERALLY SPONSORED RESEARCH

10 This invention was made in part during work supported by the U.S.  
government, including three grants from the National Institutes of Health (NIH)  
GM40781, GM31987, HL53773. The government may have certain rights in the  
invention.

TECHNICAL FIELD

15 The present invention relates generally to the field of genetic analysis.  
More specifically, this invention relates to compositions and methods for  
identifying genetic alterations of the human  $I_{1B}$ - and  $\beta_2$ -adrenergic receptors  
(ARs). The compositions and methods involve  $I_{1B}$ - and  $\beta_2$ -AR specific primer  
sets that are particularly advantageous for their ability to amplify large gene  
20 segments suitable for automated DNA sequencing analysis. Generation of these  
particular primer sets facilitates large-scale rapid screening of genetic variations  
in these receptors, which are of major physiological and clinical significance.

BACKGROUND ART

25 Adrenergic receptors (ARs) constitute a multigene family of G-protein-  
coupled receptor proteins that mediate the physiological actions of important  
neurotransmitters and hormones, including in particular, epinephrine and  
norepinephrine. ARs are broadly divided into  $\alpha$  and  $\beta$  types on the basis of their  
pharmacological specificity (R.P. Ahlquist, *Am. J. Physiol.* **153**:586-600, 1948);  
30 each class is further divided into several subtypes (e.g.  $\alpha_{1A}$ ,  $\alpha_{1B}$ ,  $\alpha_{1D}$ ;  $\alpha_{2A}$ ,  $\alpha_{2B}$ ,  $\alpha$

$\alpha_2$ ; and  $\beta_1$ ,  $\beta_2$ ,  $\beta_3$ ) according to sequence homology, drug specificity, and mechanism of signal transduction. Within this heterogeneous family, the  $\alpha_1$ -ARs play important roles in various physiological functions, including smooth muscle contraction, blood pressure homeostasis and cell growth control; whereas  $\beta_2$ -ARs play a role in regulation of cell metabolism and muscle relaxation (see e.g. P.A. Insel, *N. Engl. J. Med.* **334**:580-585, 1996; J.P. Raymond et al., *Hypertension* **15**:119-131, 1990). These subtypes of ARs are thus important targets for therapeutic agents, including drugs for cardiovascular diseases, asthma, prostatic hypertrophy and nasal congestion, among others.

As with other G-protein-coupled receptors,  $\alpha_{1B}$ -AR and  $\beta_2$ -AR generally contain a characteristic group of seven transmembrane-spanning regions, an extracellular amino terminus with ligand binding sites, and an intracellular carboxyl terminus capable of interacting with heterotrimeric G-proteins to transduce downstream signals.

Human  $I_{1B}$ -AR gene is localized to region q31-q32 on chromosome 5. It consists of two exons and a single large intron of at least 20 kilobases, which interrupts the coding region at the end of the putative sixth transmembrane domain (C.S. Ramarao et al., *J. Biol. Chem.* **267**:21936-21945, 1992). The first exon encodes two thirds of the coding sequence, and the entire sequence predicts a polypeptide of 517 amino acids with a molecular weight of about 57 kDa. Alterations in  $I_{1B}$ -AR function have been associated with disorders, such as hypertension (M.C. Michel et al., *FASEB J.* **3**:139-144, 1989), prostatic hypertrophy (K. Shibata et al., *Br. Z. Pharmacol.* **118**:1403-1408, 1996) and malignant arrhythmia in myocardial ischemia (G.E. Billman et al., *J. Cardiovas. Pharmacol.* **24**:394-402, 1994). Of particular interest are mutations resulting in constitutively active  $I_{1B}$ -AR, characterized by their marked affinity to agonists, and their ability to signal through heterotrimeric G proteins in the absence of agonist. (R.D. Guarino et al., *Cell. Signal.* **8**(5):323-333, 1996). Transgenic animals expressing altered ARs or altered levels of ARs provide a convenient means of testing for the effects of such alterations. For example, transgenic mice

expressing constitutively active I<sub>1B</sub>-ARs in their thyroid cells developed hyperthyroidism and thyroid follicular cell malignancies (C. Ledent et al., *Endocrinology* **138**(1):369-378, 1997); whereas transgenic mice deficient in I<sub>1B</sub>-AR, exhibited decreased blood pressure response and attenuated vascular contraction when stimulated with I-agonists (A. Cavalli et al. *Proc. Natl. Acad. Sci. USA*, **94**: 11589-11594, 1997).

Human  $\beta_2$ -AR is encoded by an intronless gene, mapped to the same chromosomal region in which I<sub>1B</sub>-AR gene is located (J.R. Sheppard et al., *Proc. Natl. Acad. Sci. USA* **80**:233-236, 1983). The  $\beta_2$ -AR locus is polymorphic. At least six polymorphic forms of  $\beta_2$ -AR have been identified (S.B. Liggett et al., *Clinical and Experimental Allergy* **25**(2):89-94, 1995). Exemplary polymorphisms consist of amino acid substitutions at position 16 (arginine to glycine), 27 (glutamine to glutamate), and 164 (threonine to isoleucine). Research over the past five years has strongly implicated a causal role of  $\beta_2$ -AR polymorphism in the pathogenesis of asthma. Specifically, certain polymorphisms appear to contribute to baseline hyper-reactivity, play a role in the severity of asthma, and dictate certain asthmatic phenotypes. By way of illustration, glycine 16 polymorphism has been associated with nocturnal asthma (J. Turki et al., *J. Clin. Invest.* **95**:1635-1641, 1995). The substitution of arginine with glycine apparently imparts an enhanced downregulation of the receptor, which is consistent with the nocturnal downregulation of  $\beta_2$ -AR and the diminished response to  $\beta$ -agonists observed in nocturnal asthma. The same polymorphic variant was noted in essential hypertension in African Caribbeans (P. Kontanko et al., *Hypertension* **30**(4): 773-776, 1997).

Traditionally, the identification of genetic alterations, particularly mutations and polymorphisms that are associated with disease states, has typically been proceeded by two approaches; neither of which is generally amenable for large-scale diagnostic screens or suitable for all genetic analyses. The first approach involves direct analysis of the target gene, employing techniques such as (a) direct hybridization of a gene-specific probe; (b) the use of a mutation-specific

restriction endonuclease; and (c) the use of oligonucleotide probes to detect a point mutation. Gene-specific probes are generally limited to detection of a recessive genetic disorder resulting from a deletion of part or all of the genomic DNA sequence complementary to the DNA probe. If the entire probe sequence is deleted, the homozygous condition is characterized by the complete absence of any DNA fragments that can be recognized by the probe. The second method, employing a mutation-specific restriction endonuclease, is only useful when the molecular defect responsible for the disorder abolishes or creates a new restriction enzyme recognition site. Thirdly, the use of oligonucleotide probes is generally restricted to detection of genetic alterations in which the exact sequences of the normal and mutant gene are known. To cover a spectrum of mutations causing the same disease phenotype, a battery of oligonucleotide probes is thus required. Therefore, oligonucleotide probes are not desirable for disorders that arise from previously unrecognized new mutations.

The second approach for identifying genetic alterations involves an indirect analysis by restriction fragment length polymorphism (RFLP). RFLP occurs if a variation in DNA sequence creates a new restriction enzyme site or abolishes an existing one. There are many technical disadvantages affecting the use of RFLPs. The major problem is the inability of a test to detect changes that do not affect cleavage with a restriction endonuclease. The other shortcoming is the potential for recombination between the disease gene and the polymorphic site, which render a pedigree analysis of genetic diseases uninformative. To be useful for diagnosis, a polymorphic marker must be closely linked to the site of the disease mutation. However, even when the RFLPs are only a few kilobase pairs away from the disease gene, the chance of recombination may still be quite high if a hotspot for DNA recombination is located between the markers and the mutation. Moreover, as in many of the test methods aforementioned, detection of RFLPs is expensive, labor intensive, and time consuming.

For many disorders, the location and nature of the molecular defects are unknown. Among genes that have been investigated for structural aberrations

leading to human genetic diseases, most of the sequence variants involve changes in single nucleotide positions. This is also the case for  $\alpha_{1B}$ -AR and  $\beta_2$ -AR variants identified thus far. Therefore, it appears that an ideal method for screening genetic alterations associated with clinical states should allow (a) a rapid amplification of the target gene from a complex mixture of nucleic acids; and (b) direct DNA sequencing of the resulting amplified products to determine every existing single base change.

Polymerase chain reaction (PCR) and automated nucleotide sequencing techniques can meet these criteria and have successfully been employed for the identification of abnormalities in some but not all disease genes. It is a well-known problem in the art that standard protocols for PCR are not applicable for every new application. For instance, attempts to isolate the entire coding sequence of  $\beta_2$ -AR or  $I_{1B}$ -AR gene (or even its two individual exons) from a complex mixture of genomic DNA by PCR for a sequence analysis have not until now been reported. Some often-encountered problems include lack of detectable product or a low yield of the desired product; heterogeneity of products due to mispriming or misextension; and the formation of "primer-dimers" that compete for amplification with the desired product. To increase the frequency of the desired products, further genetic manipulations such as subcloning a mixture of PCR products and screening for the desired DNA fragment are required prior to nucleotide sequencing. However, these procedures render automation of the DNA analysis of  $\alpha_{1B}$ -AR and  $\beta_2$ -AR genes much more difficult and infeasible on a large scale.

There thus remains a considerable need for compositions and methods that allow for a rapid isolation of  $\alpha_{1B}$ -AR and  $\beta_2$ -AR genes in large quantity and substantial homogeneity, which is necessary for automated sequencing analysis. The development of these compositions and methods would greatly facilitate screening for genetic abnormalities of  $I_{1B}$ -AR and  $\beta_2$ -AR that are associated with disease processes.



### SUMMARY OF THE INVENTION

The present invention provides compositions that can be used to generate a fast and specific amplification of the coding sequences of the human I<sub>1B</sub>-AR and 9<sub>2</sub>-AR genes. Specifically, the invention provides I<sub>1B</sub>-AR- and 9<sub>2</sub>-AR-specific primers capable of generating large DNA segments in substantial homogeneity. The invention also provides a method of using these primers for amplifying I<sub>1B</sub>-AR and 9<sub>2</sub>-AR gene segments, a method of using the resultant products for the identification of genetic alterations in these receptors, and a method of diagnosing diseases associated with the detected alterations in these receptor genes.

Accordingly, one embodiment of this invention is a primer pair for amplifying an I<sub>1B</sub>-AR gene which exhibits preferably a majority of the following characteristics, more preferably most of the listed characteristics, even more preferably all of the following characteristics: a) each individual primer of a pair is non-self hybridizing, contains at least 15 nucleotides, and has a melting temperature within the range of 50°C to 85°C; b) the selected pair is non-cross hybridizing; c) the selected pair anneals to two distinct regions, which are separated by a distance of at least about 400 nucleotides within the gene; d) the selected pair produces a substantially homogenous plurality of amplified fragments in a polymerase chain reaction; and e) at least one primer of the pair is capable of extending its 3' end sequence complementary to the template sequence in a DNA polymerase reaction.

In one aspect of this embodiment, an I<sub>1B</sub>-AR gene specific primer pair amplifies a fragment of exon 1 selected from the group consisting of the N-terminal region A and the C-terminal region B as shown in Figure 1. Fragment A can be amplified by a primer pair having two primers each comprising a linear sequence, preferably essentially identical to the polynucleotide shown in SEQ ID NO: 1 or 2; more preferably, it is amplified by two primers each having a linear sequence identical to the polynucleotide shown in SEQ ID NO: 1 or 2. Fragment B can be amplified by a primer pair having two primers each comprising a linear sequence, preferably essentially identical to the polynucleotide shown in SEQ ID

NO:3 or 4; more preferably, it is amplified by two primers each having a linear sequence identical to the polynucleotide shown in SEQ ID NO:3 or 4.

A further embodiment of this invention is a primer pair for amplifying  $\mathfrak{g}_2$ -AR gene, having preferably a majority of the following characteristics, more preferably most of the characteristics, even more preferably all of the following characteristics: a) each individual primer of a pair is non-self hybridizing, contains at least 15 nucleotides, and has a melting temperature within the range of 50°C to 85°C; b) the selected pair is non-cross hybridizing; c) the selected pair anneals to two distinct regions, which are separated by a distance of at least about 400 nucleotides within the gene; d) the selected pair produces a substantially homogenous plurality of amplified fragments in a polymerase chain reaction; and e) at least one primer of the pair is capable of extending its 3' end sequence complementary to the template sequence in a DNA polymerase reaction. In one aspect of this embodiment, a  $\mathfrak{g}_2$ -AR gene specific primer pair amplifies a fragment of the coding region selected from the group consisting of the N-terminal region A and the C-terminal region B as shown in Figure 2. Preferably, fragment A is amplified by a primer pair having two primers each comprising a linear sequence, preferably essentially identical to the polynucleotide shown in SEQ ID NO:5 or 6; more preferably, it is amplified by two primers each having a linear sequence identical to the polynucleotide shown in SEQ ID NO:5 or 6. Fragment B is amplified by a primer pair having two primers each comprising a linear sequence, preferably essentially identical to the polynucleotide shown in SEQ ID NO:7 or 8; more preferably, it is amplified by two primers each having a linear sequence identical to the polynucleotide shown in SEQ ID NO:7 or 8.

Another embodiment of this invention is a method of amplifying a segment of the coding sequence of  $I_{1B}$ -AR gene of a subject. Still another embodiment of this invention is a method of amplifying a segment of the coding sequence of  $\mathfrak{g}_2$ -AR gene of a subject. Each method generally comprises the steps of providing a biological sample containing nucleic acid molecules from the subject; employing a pair of  $I_{1B}$ -AR or  $\mathfrak{g}_2$ -AR gene specific primers as described

herein, a primer-dependent DNA polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of gene segments.

Another embodiment of this invention is a method of identifying a genetic variation in  $I_{1B}$ -adrenergic receptor gene of a subject. Still another embodiment of this invention is a method of identifying a genetic variation in  $\beta_2$ -AR gene. Each method generally comprises the steps of providing a biological sample containing nucleic acid molecules from the subject; amplifying a segment of the gene by employing at least one of the primer pairs of the present invention as described herein; and identifying sequence variations of the resultant products relative to a control involving one or more of the following analytical steps, including nucleotide sequencing, single-strand conformation polymorphism assay, allele-specific oligonucleotide hybridization, Southern blot analysis, and restriction endonuclease digestion.

Yet another embodiment of this invention is a method for diagnosing diseases associated with genetic alterations of  $I_{1B}$ -adrenergic receptor. A further embodiment of this invention is a method for diagnosing diseases based on genetic alterations of  $\beta_2$ -adrenergic receptor. Each method generally comprises the steps of providing a biological sample of the subject containing nucleic acid molecules; amplifying a segment of the gene by employing at least one of the primer pairs of the present invention as described herein, a primer-dependent DNA polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of amplified segments of the gene; screening the resultant segments for genetic variations relative to a control involving at least one analytical step as listed above; and determining a correlation between the detected variations in the subject and a control. Diseases resulting from alterations in  $I_{1B}$ -AR and  $\beta_2$ -AR can include cardiovascular, peripheral vascular, pulmonary, prostatic and neuro-psychic, and endocrine-metabolic disorders.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (SEQ ID NO:9) depicts the DNA sequence encoding exon 1 of the human  $\alpha_{1B}$ -adrenergic receptor gene. Nucleotide numbering starts with 1 of the noncoding sequence as published (C.S. Ramarao et al., *J. Biol. Chem.* 267:21936-21945). The coding region is shown in upper case letters. Intron and noncoding sequences are shown in lower case letters. Only a partial intron sequence is shown. Sites for potential N-linked glycosylation are indicated with triangles. Exemplary primers (SEQ ID NOS: 1-4) are shown as boxed in Figure 1. The sequence segment amplified by primers SEQ ID NOS:1 and 2 is denoted A, and that amplified by primers SEQ ID NOS:3 and 4 is denoted B.

Figure 2 (SEQ ID NO:10) depicts the DNA sequence encoding the human  $\beta_2$ -adrenergic receptor gene. Nucleotide numbering starts with 1 of the noncoding sequence as published (B.K. Kobilka et al. *Proc. Natl. Acad. Sci. USA* 84:46-50, 1987). The coding region is shown in upper case letters. The noncoding sequence is shown in lower case letters. Exemplary primers (SEQ ID NOS: 5-8) are shown as boxed in Figure 2. The sequence segment amplified by primers SEQ ID NOS:5 and 6 is denoted A, and that amplified by primers SEQ ID NOS:7 and 8 is denoted B.

Figure 3 depicts a schematic representation of the genomic organization of the human  $I_{1B}$ -AR gene. Panel A is a restriction map of the gene. The line representing the gene is interrupted at the location corresponding to the gap in the intron. Exons are indicated by solid boxes. Restriction sites are indicated by vertical lines. Panel B details the restriction sites of the coding region: B, BamHI; E, EcoRI; K, KpnI; N, NotI; P, PstI; S, SmaI; X, XhoI. Putative cyclic AMP responsive element and polyadenylation sites are indicated.

Figure 4 is a representation of an agarose gel electrophoretic separation of amplified PCR products. Human  $I_{1B}$ -AR gene segments A and B (see Figure 1) are shown in lanes 1 and 2. Human  $\beta_2$ -AR gene segments A and B (see Figure 2) are shown in lanes 3 and 4, respectively. DNA molecular weight standards are included to indicate the electrophoretic mobility of the amplified products.

Figure 5 depicts the sequence profile of fragment A of the human  $\alpha_{1B}$ -AR gene. The amplified fragments were sequenced with the amplification primer SEQ ID NO:1 using Applied Biosystems' DNA sequencer. Each peak indicates a different nucleotide. In some cases "ambiguous reads" (N) occur as the result of duplicate bases (heterozygosity) or reading ambiguities, which can generally be corrected afterwards.

Figure 6 depicts the sequence profile of fragment A of the human  $\beta_2$ -AR gene. The amplified fragments were sequenced with the amplification primer SEQ ID NO:6 using Applied Biosystems' DNA sequencer. Each peak indicates a different nucleotide. In some cases "ambiguous reads" (N) occur as the result of duplicate bases (heterozygosity) or reading ambiguities, which can generally be corrected afterwards.

#### DETAILED DESCRIPTION OF THE INVENTION

As noted above, this invention provides compositions and methods for a rapid amplification of the coding sequence of a  $\alpha_{1B}$ -AR gene or a  $\beta_2$ -AR gene, to generate a population of substantially homogenous gene segments. The amplified gene segments can be directly used for a high throughput screen of sequence variants, e.g., by automated DNA sequencing analysis. Accordingly, the invention provides a method of identifying genetic alterations of  $\alpha_{1B}$ -AR or  $\beta_2$ -AR by detecting changes in the gene nucleotide sequences. The invention further provides a method for diagnosing diseases resulting from genetic alterations of these receptors.

#### Definitions

The term "polynucleotide" or "nucleic acid" refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or

analogous thereof. The terms "polynucleotide" and "nucleotide" as used herein are used interchangeably. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. A "fragment" or "segment" of a nucleic acid is a small piece of that nucleic acid.

A "gene" refers to a polynucleotide containing at least one open reading frame that is capable of encoding a particular protein after being transcribed and translated.

A "primer" is a short polynucleotide, generally with a free 3' -OH group, that binds to a target or "template" potentially present in a sample of interest by hybridizing with the target, and thereafter promoting polymerization of a polynucleotide complementary to the target. A "polymerase chain reaction" ("PCR") is a reaction in which replicate copies are made of a target polynucleotide using a "pair of primers" or a "set of primers" consisting of an "upstream" and a "downstream" primer, and a catalyst of polymerization, such as a DNA polymerase, and typically a thermally-stable polymerase enzyme. Methods for PCR are well known in the art, and taught, for example in "PCR: a practical approach" (M. MacPherson et al., IRL Press at Oxford University Press 1991). All processes of producing replicate copies of a polynucleotide, such as PCR or gene cloning, are collectively referred to herein as "replication."

In the context of polynucleotides, a "linear sequence" or a "sequence" is an order of nucleotides in a polynucleotide in a 5' to 3' direction in which residues that neighbor each other in the sequence are contiguous in the primary structure of the polynucleotide.

5 "Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson-Crick base pairing, Hoogsteen binding, or in any other sequence-specific manner. The complex may comprise two strands forming a duplex structure, three or more  
10 strands forming a multi-stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of a PCR reaction, or the enzymatic cleavage of a polynucleotide by a ribozyme.

Hybridization reactions can be performed under conditions of different  
15 "stringency". Relevant conditions include temperature, ionic strength, time of incubation, the presence of additional solutes in the reaction mixture such as formamide, and the washing procedure. Higher stringency conditions are those conditions, such as higher temperature and lower sodium ion concentration, which require higher minimum complementarity between hybridizing elements  
20 for a stable hybridization complex to form. Conditions that increase the stringency of a hybridization reaction are widely known and published in the art: see, for example, "Molecular Cloning: A Laboratory Manual", Second Edition (Sambrook, Fritsch & Maniatis, 1989).

When hybridization occurs in an antiparallel configuration between two  
25 single-stranded polynucleotides, the reaction is called "annealing" and those polynucleotides are described as "complementary". A double-stranded polynucleotide can be "complementary" or "homologous" to another polynucleotide, if hybridization can occur between one of the strands of the first polynucleotide and the second. "Complementarity" or "homology" (the degree  
30 that one polynucleotide is complementary with another) is quantifiable in terms of

the proportion of bases in opposing strands that are expected to form hydrogen bonding with each other, according to generally accepted base-pairing rules.

“Melting temperature” of a primer refers to the temperature at which 50% of the primer-template duplexes are dissociated. Melting temperature is a function of ionic strength, base composition, and the length of the primer. It can be empirically determined, e.g., by measuring shift in optical density or can also be predicted using either of the following equations:

$$T_m (^{\circ}\text{C}) = 81.5 + 16.6 \times \log [\text{Na}] + 0.41 \times (\% \text{GC}) - 600/N$$

where [Na] is the concentration of sodium ions, and the % GC is in number percent, where N is chain length, or

$$T_m (^{\circ}\text{C}) = 2 \times (\text{A}+\text{T}) + 4 \times (\text{C}+\text{G})$$

where A, T, G and C represent the number of adenosine, thymidine, guanosine and cytosine residues in the primer.

“Denaturation” refers to the process by which a double-stranded nucleic acid is converted into its constituent single strands. Denaturation can be achieved, for example, by the use of high temperature, low ionic strength, acidic or alkaline pH, and/or certain organic solvents. Methods for denaturing nucleic acids are well known in the art.

“Non-self hybridizing” as applied to a primer, means that the primer is essentially incapable of forming intra-molecular duplex mediated by hydrogen-bonding between the bases of the nucleotide residues within the primer under hybridization conditions of high stringency. A “non-self hybridizing” primer is essentially free of intra-molecular duplex preferably under a high stringency condition, more preferably under a moderately stringent condition, even more preferably under a low stringent condition. Factors determining hybridization stringency are well known in the art, and exemplary conditions of different stringency are provided in the descriptions that follow. The term “non-cross hybridizing” as applied to primer pairs means that the two individual primers do not hybridize and form a duplex stabilized by hydrogen-bonding between



complementary bases in the two primers under hybridization conditions of high stringency.

In determining whether a primer is non-self hybridizing or a primer pair is non-cross hybridizing, sequence complementarity within the primer and between  
5 the primers should be examined. A non-self hybridizing primer is one that lacks internal sequence homology necessary for duplex formation. Similarly, a pair of non-cross hybridizing primers does not share a sufficient amount of sequence homology to form a stable double-stranded structure.

A linear sequence of nucleotides is "identical" to another linear sequence,  
10 if the order of nucleotides in each sequence is the same, and occurs without substitution, deletion, or material substitution. It is understood that purine and pyrimidine nitrogenous bases with similar structures can be functionally equivalent in terms of Watson-Crick base-pairing; and the inter-substitution of like nitrogenous bases, particularly uracil and thymine, or the modification of  
15 nitrogenous bases, such as by methylation, does not constitute a material substitution. An RNA and a DNA polynucleotide have identical sequences when the sequence for the RNA reflects the order of nitrogenous bases in the polyribonucleotides, the sequence for the DNA reflects the order of nitrogenous bases in the polydeoxyribonucleotides, and the two sequences satisfy the other  
20 requirements of this definition. Where one or both of the polynucleotides being compared is double-stranded, the sequences are identical if one strand of the first polynucleotide is identical with one strand of the second polynucleotide.

A linear sequence of nucleotides is "essentially identical" to another linear sequence, if both sequences are capable of hybridizing to form a duplex with the  
25 same complementary polynucleotide. Sequences that hybridize under conditions of greater stringency are more preferred. It is understood that hybridization reactions can accommodate insertions, deletions, and substitutions in the nucleotide sequence. Thus, linear sequences of nucleotides can be essentially identical even if some of the nucleotide residues do not precisely correspond or  
30 align. In general, essentially identical sequences of about 40 nucleotides in length

will hybridize at about 40°C in 10X SSC (low stringency) or solution with equivalent ionic strength / temperature; preferably, they will hybridize at about 40 °C in 6 x SSC; more preferably, they will hybridize at about 50 °C in 6 x SSC (moderate stringency); even more preferably, they will hybridize at about 60 °C in 6 x SSC, or at about 40 °C in 0.5 x SSC, or at about 30 °C in 6 x SSC containing 50% formamide; still more preferably, they will hybridize at 40 °C or higher in 2 x SSC or lower in the presence of 50% or more formamide or equivalent ionic strength / temperature (high stringency). It is understood that the rigor of the test is partly a function of the length of the polynucleotide; hence shorter polynucleotides with the same homology should be tested under lower stringency and longer polynucleotides should be tested under higher stringency, adjusting the conditions accordingly. The relationship between hybridization stringency, degree of sequence identity, and polynucleotide length is known in the art and can be calculated by standard formulae. Sequences that correspond or align more closely to the invention disclosed herein are comparably more preferred. Generally, essentially identical sequences are at least about 80% identical with each other, after alignment of the homologous regions. Preferably, the sequences are at least about 85% identical; more preferably, they are at least about 90% identical; more preferably, they are at least about 95% identical; still more preferably, the sequences are 100% identical.

In determining whether nucleic acid sequences are essentially identical, a sequence that preserves the functionality of the nucleic acid with which it is being compared is particularly preferred. Functionality may be established by different criteria, such as ability to hybridize with a target polynucleotide, ability to effectively amplify a target sequence to yield a substantially homogenous multiplicity of products, and the ability to extend the 3' end sequence complementary to a target sequence in a DNA polymerase reaction, such as a nucleotide sequencing reaction. Primers having linear sequences that are essentially identical to those of the exemplary primers are considered as "derivatives".

As used herein, a "substantially homogenous plurality" of amplified products refers to a mixture of DNA fragments in which the undesired DNA fragments as indicated by, e.g., unexpected molecular sizes, constitute less than about 25% of the total amount of products. Preferably, the undesired fragments constitute less than 20%, more preferably 15% and even more preferably 10%. Product homogeneity may be indicated by a number of means, such as agarose gel electrophoresis of the amplified products, followed by visualizing a single DNA band upon staining the gel. Product homogeneity may also be determined by quantitative Southern blot analysis using the same primer pair employed in the amplification as probe. A ratio of 75% or above between the intensities of the band of predicted size and the bands of unexpected sizes indicates that the amplified products are substantially homogenous. Preferably, the ratio is above 80%, more preferably it is above 85%, even more preferably it is above 90%, still more preferably it is above 95%.

A "subject" or an "individual" refers to a human being.

A "genetic alteration" or "genetic variation" refers to a mutation or polymorphism in the nucleotide sequence of a given polynucleotide that may result in subsequent changes in the amino acid sequence of the encoded polypeptides. Mutations may arise from insertion, deletion, substitution or translocation of one or more nucleotide residues. A subject carries an altered or variant receptor gene when a difference in nucleotide sequence relative to that of a control is observed. A "control" is an alternative subject or sample used in an experiment for comparison purpose. A control can be "positive" or "negative". For example, where the purpose of the experiment is to determine a correlation of certain genetic alterations with a particular disease, it is generally preferable to use a positive control (a subject or a sample from a subject, carrying such a genetic alteration and exhibiting syndromes characteristic of that disease), and a negative control (a subject or a sample from a subject lacking the genetic alteration and clinical syndrome of that disease).

Selection of  $\alpha_{1B}$ -AR or  $\beta_2$ -AR gene specific primers

A central aspect of the invention is the design of DNA primer pairs capable of amplifying large fragments of  $\alpha_{1B}$ - or  $\beta_2$ -AR coding sequences from a complex mixture of nucleic acids to yield a substantially homogenous population of products that can be directly analyzed by automated nucleotide sequencing. The primer pairs of the present invention exhibit preferably a majority of the following characteristics, more preferably most of the characteristics, and even more preferably all of the characteristics as listed below:

- a) each individual primer of a pair is non-self hybridizing, contains at least 15 nucleotides, and has a melting temperature within the range of 50°C to 85°C;
- b) the selected pair is non-cross hybridizing;
- c) the selected pair anneals to two distinct regions of the receptor gene, and said regions are separated by a distance of at least about 400 nucleotides;
- d) the selected pair produces a substantially homogenous plurality of amplified fragments in a polymerase chain reaction; and
- e) at least one primer of the pair is capable of extending its 3' end sequence complementary to the template sequence in a DNA polymerase reaction.

Several factors apply to the design of primer pairs having the above-mentioned characteristics. Firstly, the selected primer pair should contain sequences that are specific to the receptor genes and unique to the entire genome. Secondly, the two opposing primers of a pair should have similar thermal profiles and internal stability. This can be achieved by selecting primers with comparable length and G/C content. Preferred primers are from 18 to 23 nucleotide in length, having 50 to 60% G+C composition. In addition, the two opposing primers should not contain sequences that share internal homology or homology to each other. Extensive homology within the primer, e.g. due to inverted repeats,

promotes self-hybridization; complementarity of the two primers to each other, especially at their 3' ends, can cause cross-hybridization of primers and the generation of primer-dimers.

In a preferred embodiment, a primer pair generates a large fragment of the  $I_{IB}$ -AR gene, encompassing either the N-terminal half of the coding sequences of exon 1 as shown in region A of Figure 1, or the C-terminal half of the coding sequences as shown in region B of Figure 1. Preferred primer pairs replicate fragments of at least about 400 nucleotides within region A or B. Applying these criteria, by way of illustration, an exemplary primer pair that amplifies the N-terminal fragment of the  $I_{IB}$ -AR gene includes an upstream and a downstream primer, having the nucleotide sequence 5'-CGGGGGAAGCAAAGTTTCA-3' (SEQ ID NO:1) and 5'-CGGCAGTACATGACTAGAAT-3' (SEQ ID NO:2), respectively. Another exemplary pair of primers that amplifies the C-terminal fragment consists of two opposing primers having the nucleotide sequence 5'-CTCTCCTTGGGTGGAAGGA-3' (SEQ ID NO:3) and 5'-AGCTCATCAGTAAACCCAAG-3' (SEQ ID NO:4), respectively. The resulting N-terminal fragment contains 766 nucleotide bases, and the C-terminal fragment has 473 nucleotide bases. Also encompassed by the invention are primers essentially identical to the two aforementioned primer sets which are selected to meet the criteria described above. An essentially identical primer set retains the characteristics and functionalities of the two exemplified primer pairs, even if they may contain mismatched nucleotide sequences. It is known in the art that a "perfectly matched" primer is not needed for a specific amplification. Minor changes in primer sequence achieved by substitution, deletion or insertion of a small number of bases do not affect the amplification specificity. For instance, primers may contain 5' extensions or mismatches for incorporating restriction enzyme sites, an ATG start codon, or promoter sequences into the target sequence. Mismatched bases can also be placed internally. In general, as much as 20% base-pair mismatch (when optimally aligned) can be tolerated. Changes in nucleotide residues positioned at

the 5' end of a primer is preferred over changes of that at the 3' end. Alteration of nucleotides located in the 3' end is preferred over changes in the middle of the primer.

In another preferred embodiment, a primer pair generates a large fragment of  $\beta_2$ -AR gene, encompassing either the N-terminal half of the coding sequences as shown in region A of Figure 2, or the C-terminal half of the coding sequences as shown in region B of Figure 2. Preferred primer pairs replicate fragments of at least about 400 nucleotides within region A or B. An exemplary primer pair that amplifies the N-terminal fragment includes an upstream and a downstream primer, having the nucleotide sequence 5'-GAATGAGGCTTCCAGGCGTC-3' (SEQ ID NO:5) and 5'-GATGATGCCTAACGTCTTG-3' (SEQ ID NO:6), respectively. The other exemplary pair of primers that amplifies the C-terminal fragment consists of two opposing primers having the nucleotide sequence 5'-TTCTACGTGCCCCTG GTG-3' (SEQ ID NO:7) and 5'-TCCTCTAGGACTAAAGCTC-3' (SEQ ID NO:8), respectively. The resulting N-terminal fragment contains 917 nucleotide bases, and the C-terminal fragment has approximately 862 nucleotide bases. Also encompassed by the invention are primers essentially identical to the above-described two primer pairs.

Primers of the present invention can contain modified nucleotides, such as those conjugated with fluorophores, radioactive labels, and various chemical couplers including but not limited to biotin, alkaline phosphatase, horseradish peroxidase and digoxigenin. Primers provided by the invention may be either phosphorylated or non-phosphorylated at their 5' ends. It is within the skill of the art to prepare either type of primer by automated synthesis (see, e.g., Applied Biosystems Model 380D DNA synthesizer User's Manual). Phosphorylated oligonucleotides are synthesized on an automated instrument by using a phosphorylated monomer in the final coupling cycle. Such a phosphorylated monomer for use in automated synthesis may be obtained commercially, for example from Clontech Laboratories, Palo Alto, CA or Applied Biosystems, Foster City, CA or Glen Research, Sterling, VA or other commercial vendors.

Alternatively, a phosphorylated oligonucleotide may be prepared through automated synthesis of a 5'-hydroxyl-terminated oligonucleotide, followed by enzymatic phosphorylation using methods well-known in the art (e.g., Maniatis et al., Sambrook et al., Ausubel et al., supra). Preferably, synthesized primers are  
5 purified prior to gene amplification.

#### Uses of the human $I_{1B}$ -AR and $\beta_2$ -AR gene specific primer sets

Use of the primer sets provided by this invention provides rapid amplification of the coding sequences of  $I_{1B}$ -AR or  $\beta_2$ -AR gene to yield discrete  
10 gene segments in large quantity and substantial homogeneity. The amplified products are suitable for direct sequence analysis for identifying variations in nucleotide sequences, and therefore for diagnosing genetic diseases based on the altered sequences. The amplified products can also be used for generating receptor proteins for structure determination, to assay a molecule's activity on  
15 these receptors, and to screen for receptor-interacting molecules useful as diagnostics and/or therapeutics.

#### *Amplification of the human $I_{1B}$ -AR and $\beta_2$ -AR genes*

In one embodiment, this invention provides a method for amplifying the coding sequence of exon 1 in the  $I_{1B}$ -AR gene of a subject. In another  
20 embodiment, the invention provides a method of amplifying the entire coding sequence of  $\beta_2$ -AR gene. Each method generally comprises the following steps:  
a) providing a biological sample containing nucleic acid molecules from the subject; b) employing a primer pair as described herein, a primer-dependent  
25 polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of gene segments in a polymerase reaction.

Biological samples used for this invention encompass body fluid, solid tissue samples, tissue cultures or cells derived therefrom and the progeny thereof, and sections or smears prepared from any of these sources, or any other samples  
30 expected to contain genetic material of the subject. Nucleic acid contained in

these samples can be extracted according to standard methods in the art, e.g., as illustrated herein (Example 3). For instance, DNA and RNA can be isolated using various lytic enzymes or chemical solutions according to the procedures set forth in Sambrook et al. ("Molecular Cloning: A Laboratory Manual", Second Edition, 1989), or extracted by nucleic-acid-binding resins following the accompanying instructions provided by manufactures.

For the purpose of this invention, amplification means any method employing a primer-dependent polymerase capable of replicating a target sequence with reasonable fidelity. Amplification may be carried out by natural or recombinant DNA-polymerases such as T7 DNA polymerase, Klenow fragment of E. coli DNA polymerase, and reverse transcriptase.

A preferred amplification method is PCR. General procedures for PCR are taught in "PCR: a practical approach" (M. MacPherson et al., IRL Press at Oxford University Press 1991). However, PCR conditions used for each application reaction are empirically determined. A number of parameters influence the success of a reaction. Among them are annealing temperature and time, extension time,  $Mg^{2+}$  ATP concentration, pH, and the relative concentration of primers, templates, and deoxyribonucleotides. Provided herein are conditions that are optimized for replicating the human  $I_{1B}$ -AR and  $\beta_2$ -AR gene with the aforementioned primer pairs. Preferably, the template nucleic acids are denatured by heating to at least about 90°C prior to the polymerase reaction. Typically, approximately 30 cycles of amplification are executed using denaturation at a range of 90°C to 95°C for 0.5 to 1 minute, annealing at a temperature ranging from 55°C to 65°C for 1 to 2 minutes, and extension at 68°C to 75°C for at least 1 minute with the final cycle extended to 10 minutes. Each PCR reaction typically contains 100 ng template nucleic acids, 1  $\mu$ M of upstream and downstream primers, and 0.1-0.2 mM dNTP of each kind, and 1 to 5 units of commercially available DNA polymerases.

After amplification, the resulting DNA fragments can be detected by agarose gel electrophoresis followed by visualization with ethidium bromide



staining and ultraviolet illumination. A specific amplification of the receptor gene can be verified by demonstrating that the amplified DNA fragment has the predicted size, exhibits the predicated restriction digestion pattern, and/or hybridizes to the correct cloned DNA sequence.

5

*Detection of genetic variations in the human I<sub>1B</sub>-AR or  $\mathfrak{I}_2$ -AR*

Whereas there is a clear association between receptor dysfunction and various disorders, the search for the genetic basis for abnormalities of I<sub>1B</sub>-AR or  $\mathfrak{I}_2$ -AR

10

has been hampered by the continued lack of a rapid, informative and cost-effective methods applicable for analyzing a large volume of clinical samples. Without a large-scale screen of I<sub>1B</sub>-AR and  $\mathfrak{I}_2$ -AR genes obtained from normal and diseased individuals, it is difficult to establish the clinical significance of any detected alterations in these receptors.

15

Accordingly, this invention provides a method for a rapid detection of genetic variations in I<sub>1B</sub>-adrenergic receptor of a subject, and also a method of detecting genetic variations in  $\mathfrak{I}_2$ -AR. Each method generally comprises the following steps: a) providing a biological sample containing nucleic acid molecules from the subject; b) amplifying a segment of the gene by a primer pair as described above; and c) identifying sequence variations of the resulting amplified products relative to a control, e.g., by employing one or more of the following analytical steps: nucleotide sequencing, single-strand conformation polymorphism assay, allele-specific oligonucleotide hybridization, Southern blot analysis, and restriction endonuclease digestion.

20

25

In the method of the present invention, the test nucleic acid can be obtained from any of a variety of sources. For example, the test nucleic acid can be prepared from cells within a body fluid of the subject or from cells constituting a body tissue of the subject.

30

The particular body fluid from which the cells are obtained can be selected from the group consisting of blood, pleural fluid, ascites, urine and spinal fluid.

Furthermore, the particular body tissue from which the cells are obtained can be selected from the group consisting of skin, endometrial, intrauterine, oral mucosa and cervical tissue. Both normal and tumor tissues can be used.

When assaying for genetic variations of I<sub>1B</sub>-AR or 9<sub>2</sub>-AR, the test nucleic acid can be, e.g. genomic DNA, cDNA or mRNA, depending on the type of sequence variants that is to be determined. To identify alternatively spliced variants, mRNA should be used. However, when examining polymorphic alleles of the receptor genes in different individuals, the test nucleic acid is preferably genomic DNA. When detecting point mutations, the preferred test nucleic acid is also genomic DNA. Genomic DNA can be prepared, e.g. as described by Sambrook et al. ("Molecular Cloning: A Laboratory Manual", Second Edition, 1989) or using commercial kits following the manufacturers' instructions. cDNA and mRNA can be isolated according to standard techniques in the art.

Amplification of the nucleic acids encoding receptor genes can be carried out by any means employing a primer-dependent polymerase known in the art. Such polymerase includes natural or recombinant DNA-polymerases. A preferred amplification method is PCR. In addition to the benefits of simplicity and efficiency, PCR using the primer sets provided herein generates a large amount of gene segments that can be directly used for subsequent DNA analyses without further genetic manipulation of the amplified products.

A variety of analytical techniques are available in the art for detecting or confirming sequence variations in a polynucleotide. The choice of a particular technique depends on the nature of the variations. Restriction endonuclease digestion can be employed to detect a sequence variation that creates or abolishes a restriction enzyme recognition site. Single-strand conformation polymorphism assay can be used for the detection of single base substitutions. Oria et al., *Proc. Natl. Acad. Sci. USA* 86:2766-2770 (1989); Gaudamo et al., *Proc. Natl. Acad. Sci. USA* 88: 5413-5417. This method is based on the observation that changes in the nucleotide sequence affect single-strand conformation, and thus resulting in altered electrophoretic mobility. In addition, allele-specific oligonucleotides can

be employed in a hybridization procedure to differentiate polymorphic alleles that carry changes in single nucleotide positions. This procedure can be performed when the sequence variations are known. Furthermore, Southern blot analysis can be used alone or in conjunction with the above-described methods to detect changes in hybridization pattern with a gene specific probe.

A preferred method of detecting genetic variations in  $I_{1B}$ -AR and  $\mathcal{Q}_2$ -AR gene is by sequencing the amplified products. Since the location and nature of the alterations are often unknown, and most of the aberrant  $I_{1B}$ -ARs and  $\mathcal{Q}_2$ -ARs identified thus far carry changes in single amino acid residues, it is desirable to examine the nucleotide sequence of the amplified products. Distinguished from the previously reported sequencing analyses involving nested primers (C.S. Ramarao et al., *J. Biol. Chem.* **267**:21936-21945, 1992; J. Turki et al., *J. Clin. Invest.* **95**:1635-1641, 1995), sequencing the amplified products of the present invention is performed using the same primer employed in the amplification reaction (see Example 5). Nested primers are generally required for obtaining an accurate reading of the nucleotide sequence because of a frequently-encountered problem, namely product heterogeneity as a result of mispriming or misextension of the amplification primers. The method of this invention does not suffer from the problems associated with nonspecific amplification. Primers of the present invention amplify specific gene segments, and provide reliable and reproducible reading of the nucleotide sequences. DNA sequencing is preferably carried out by an automated sequencer (e.g. Applied Biosystems, model 377), which reads at least 400 bases per reaction. DNA sequences can also be determined by methods employing gene chip technology, such as that offered by Affymetrix. Furthermore, sequencing can be performed manually using commercial kits (e.g. UBI) according to manufacturer's instructions.

In carrying out the aforementioned DNA analyses for determining sequence variations of the receptor genes in a subject or in a population of interest, one generally includes appropriate controls for a comparison. It is often preferable to conduct the analyses on the test sample and on the control samples

in parallel. Since some mutations and polymorphisms occur more frequently than others, a common frame of reference is preferably the receptor gene as that reported in the original cloning (C.S. Ramarao et al., *J. Biol. Chem.* **267**:21936-21945, 1992; B.K. Kobilka et al. *Proc. Natl. Acad. Sci. USA* **84**:46-50, 1987). To determine whether the polymorphisms and/or mutations detected in the test subject are homozygous or heterozygous, it is preferable to include controls whose genotypes with respect to those variations have been previously determined. Further details and alternatives for control samples are provided in the descriptions that follow.

*Diagnosing diseases that are associated with genetic alterations of human I<sub>1B</sub>-AR or  $\beta_2$ -AR*

It has been known for almost 50 years that there is an association between dysfunctions of I<sub>1B</sub>-AR and  $\beta_2$ -AR and various disorders. In recent years, a wealth of evidence has emerged that implicates a causal role of certain mutations and polymorphisms of these receptor genes in certain types of asthma (J. Turki et al., *J. Clin. Invest.* **95**:1635-1641, 1995), hypertension (L.P. Svetkey et al., *Hypertension* **27**: 1210-1215, 1996) and other cardiovascular disorders. Ohe et al. reported that Ban-I RFLP of  $\beta_2$ -AR gene is associated with bronchial asthma (M. Ohe et al. *Thorax* **50**: 353-359, 1995). Liggett et al. have shown that the polymorphic variant encoding glycine instead of arginine at position 16 is overrepresented in patients with nocturnal asthma (S.B. Liggett et al., *Clinical and Experimental Allergy* **25**(2): 89-94, 1995). Subsequent studies involving site-directed mutagenesis and recombinant expression revealed that the Glycine 16 variant exhibits an augmented receptor downregulation, which is consistent with the nocturnal downregulation of  $\beta_2$ -AR and the attenuated response to agonists observed in nocturnal asthma. More recently, the same polymorphic variant was found to be associated with hypertension. The altered receptor is likely to be responsible for the attenuated vasodilation and increase in blood pressure

commonly found in hypertensives (P. Kontanko et al., *Hypertension* **30**(4): 773-776, 1997).

An involvement of I-ARs in hypertension has also been noted. In fact, I-AR antagonists are widely used as anti-hypertensive drugs. Recent studies with  
5 I<sub>1B</sub>-AR-deficient transgenic mice have demonstrated that within the I-AR family, I<sub>1B</sub>-AR in particular, mediates agonist-induced blood pressure and vascular contractile responses (A. Cavalli et al. *Proc. Natl. Acad. Sci. USA*, **94**: 11589-11594, 1997). Therefore, it is highly likely that genetic alterations of I<sub>1B</sub>-AR leading to enhanced vasoconstrictions, and/or genetic alterations of  $\beta_2$ -AR  
10 resulting in attenuated vasodilation, contribute to human hypertension.

Accordingly, this invention provides a simple and rapid method for diagnosing diseases associated with genetic alterations of I<sub>1B</sub>-adrenergic receptors. This invention also provides a method for diagnosing diseases associated with genetic alterations of  $\beta_2$ -adrenergic receptors. Each method generally comprises  
15 the following steps: a) providing a biological sample of the subject containing nucleic acid molecules; b) amplifying a segment of the gene by employing a pair of DNA primer described herein, a primer-dependent DNA polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of amplified segments of the gene; c) screening the resulting segments for genetic variations  
20 relative to a control involving at least one analytical step as described herein; and d) determining a correlation of the detected variations between the subject and a control.

For the purpose of this invention, a nucleic acid-containing sample is any sample containing nucleic acid suitable for use in an amplification procedure. As  
25 used herein, amplification means any method for replicating a nucleic acid with the use of a primer-dependent polymerase. In a preferred embodiment, the amplification is effected by means of PCR using two sets of I<sub>1B</sub>-AR or  $\beta_2$ -AR gene specific primers as described above. Amplified products are subjected to one or more of the following analyses, including nucleotide sequencing, single-  
30 strand conformation polymorphism assay, allele-specific oligonucleotide

hybridization, Southern blot analysis, and restriction endonuclease digestion. A preferred analysis is direct nucleotide sequencing.

In diagnosing diseases associated with genetic alterations in  $I_{IB}$ -AR or  $\beta_2$ -AR, one typically conducts a comparative analysis of the subject and appropriate controls. Preferably, a diagnostic test includes a control sample derived from a subject (hereinafter positive control), that carries the same variant receptor gene and exhibits syndromes that are characteristic of the disease of interest. More preferably, a diagnosis also includes a control sample derived from a subject (hereinafter negative control), that retains the "wild-type receptor gene" (having the nucleic acid sequence as that reported in the original cloning) and lacks the clinical syndromes. A positive correlation between the subject and the positive control with respect to the identified alterations indicates the presence or a predisposition of said disease. A lack of correlation between the subject and the negative control confirms the diagnosis.

In a preferred embodiment, the method is used for diagnosing nocturnal asthma based on glycine 16 polymorphism in  $\beta_2$ -AR gene. In another embodiment, the method is used for a diagnosis of essential hypertension based on the same genetic polymorphism. While assaying for glycine 16 polymorphism, genomic DNA of the test subject can be obtained from a blood sample. The N-terminal fragment A (as shown in Figure 2) that encompasses sequences encoding the residue glycine 16 can be amplified using primers SEQ ID NOS:5 and 6, or derivatives thereof. Direct sequencing of the amplified products using the same primers employed in the amplification procedure may be performed to detect the single base change (adenosine to guanosine) at position 46 (numbering from the start codon) that results in amino acid substitution of arginine with glycine. Homozygosity of glycine 16 indicates the presence or a predisposition to nocturnal asthma and/or essential hypertension. Glycine 16 homozygotes will be apparent by comparing the sequence peaks from the subject sample to those of the controls (for an example of the sequence output from an automated DNA sequencer, see Figure 5 or 6). A single peak corresponding to

nucleotide guanosine at position 46 is indicative of glycine 16 homozygosity. Two overlapping peaks, each representing guanosine or adenosine respectively at position 46, suggest heterozygosity of glycine 16. Finally, a single peak representing adenosine shows that the subject is homozygous in arginine 16.

5

The following Examples are provided to further assist those of ordinary skill in the art. Such examples are intended to be illustrative and therefore should not be regarded as limiting the invention. A number of exemplary modifications and variations are described in this application and others will become apparent to those of skill in this art. Such variations are considered to fall within the scope of the invention as described and claimed herein.

10

## EXAMPLES

### Example 1

15

#### Generation of $\alpha_{1B}$ -AR gene specific primer sets

As an illustration of the primer sets for use in the present invention, we have designed two sets of primers (having the properties described above) that amplify two overlapping fragments encompassing the entire exon 1 sequence of the human  $\alpha_{1B}$ -AR gene. A first exemplary set contains two opposing primers each having the nucleotide sequence 5'-CGGGGGAAGCAAAGTTTCA-3' (SEQ ID NO:1) or

20

5'-CGGCAGTACATGACTAGAAT-3' (SEQ ID NO:2). A second exemplary set consists of an upstream and downstream primer having the nucleotide sequences

25

5'-CTCTCCTTGGGTGGA AGGA-3' (SEQ ID NO:3) and 5'-AGCTCATCAGTAAACCCAAG-3' (SEQ ID NO:4), respectively. Individual primers of each set are (a) non-self and non-cross hybridizing, (b) having a preferred length of 18 to 20 nucleotides, and (c) a comparable melting temperature in the range of 54 to 60 °C. The first primer set amplifies the N-

terminal half of exon 1 (fragment A in Figure 1), and yields a population of substantially homogeneous products as evidenced by a single DNA band of predicted size on an electrophoretic agarose gel (Lane 1, Figure 4). The second primer set amplifies the C-terminal half of exon 1 (fragment B in Figure 1),  
5 generating a discrete fragment of 473 base pairs as shown in Lane 2 of Figure 4. The resulting products were substantially homogenous, and thus suitable for direct sequencing analysis (see Example 5). In addition, primers of these sets, namely primer SEQ ID NOS: 1, 2 and 3 are capable of extending their 3' end sequences complementary to a template in a DNA polymerase reaction, such as a  
10 sequencing reaction. An exemplary sequence output of fragment A generated by an automated sequencer using primer SEQ ID NO: 1 is depicted in Figure 5.

### Example 2

#### Generation of $\beta_2$ -AR gene specific primer sets

15 Exemplary primer sets specific for  $\beta_2$ -AR gene were also designed to exhibit each of the characteristics listed above. Exemplary set 1 contains two primers each having a nucleotide sequence 5'-GAATGAGGCTTCCAGGCGTC-3' (SEQ ID NO:5) or 5'-GATGATGCCTAACGTCTTG-3' (SEQ ID NO:6).  
20 Exemplary set 2 consists of two primers having the nucleotide sequences 5'-TTCTACGTGCCCCTG GTG-3' (SEQ ID NO:7) and 5'-TCCTCTAGGACTAAAGCTC-3' (SEQ ID NO:8), respectively. Whereas the first set amplifies the N-terminal half of the coding sequences as shown in region A of Figure 2, resulting in a large fragment of 917 nucleotide bases; the second  
25 set amplifies the C-terminal half of the coding sequences, yielding a fragment of 862 base pairs. The products generated by these primer sets are of substantial homogeneity as reflected by discrete bands of predicted sizes on an electrophoretic agarose gel (Lanes 3 and 4 in Figure 4). Furthermore, at least one primer of each set, namely primer SEQ ID NOS:5, 6 and 7, can be used for direct  
30 sequencing of the amplified fragments. An exemplary sequence profile of



fragment A, which was determined by an automated sequencer using primer SEQ ID NO:6 is shown in Figure 6.

### Example 3

5     Use of  $\alpha_{1B}$ -AR primer sets to amplify  $\alpha_{1B}$ -AR coding sequence from human genomic DNA

Approximately 3ml of heparinized human blood was drawn from healthy volunteers. Genomic DNA was extracted using a commercial DNA Isolation Kit  
10     (Gentra System, Inc.) according to accompanying instructions.  $\alpha_{1B}$ -AR primer sets 1 and 2 (see Table 1) were used to amplify the coding sequence of exon 1 by PCR.

PCR was performed in a 100  $\mu$ l reaction mixture containing 100ng of purified genomic DNA, 1 micromole of upstream and downstream primers, 2.5  
15     mM  $MgCl_2$ , 50 mM NaCl, 10 mM Tris-Cl, pH 8.3, 0.2 mM each dNTP (Pharmacia), and 5 units of Amplitaq Gold Polymerase (Perkin Elmer). The reaction mixture was initially incubated at 95 °C for 10 minutes and then subjected to 35 cycles of PCR in a Perkin-Elmer/Cetus thermocycler as follows:

denaturation 94°C, 30 seconds  
20     annealing 60°C, 1.5 minutes  
extension 72°C, 1.5 minutes - final extension 10 minutes.

After amplification, the products from each amplification reaction were resolved by electrophoresis on a 1% Seakem agarose gel, and then visualized upon staining with ethidium bromide. As shown in lanes 1 and 2 of Figure 4,  
25     each primer set generated a discrete DNA band of predicted size.

#### Example 4

##### Use of $\beta_2$ -AR primer sets to amplify $\beta_2$ -AR coding sequence from human genomic DNA

5 Human genomic DNA was prepared from a blood sample as described above. Two sets of  $\beta_2$ -AR primers were used to amplify two overlapping fragments that encompass the entire coding sequence of  $\beta_2$ -AR gene (see Table 1). Amplification was performed by PCR under the same conditions as described in Example 3. The resultant products were separated on an agarose gel. As  
10 indicated in lanes 3 and 4 of Figure 4, the two primer sets generated discrete fragments of approximately 917 base pairs and 862 base pairs, respectively.

#### Example 5

##### Sequence Analysis

15 The PCR products of expected sizes were cut from the gel and the DNA was purified using QIAquick Gel Extraction Kit. The extracted DNA was resuspended in Tris-EDTA buffer (10mM Tris-Cl, 1mM EDTA, pH 8.0) and concentrated using a Centricon Concentrator (Amicon). The purified gene fragments were then sequenced by an automated DNA sequencer (Applied  
20 Biosystems, model 377) using one or more of the same primers employed in PCR. The upstream primer SEQ ID NO:1 and the downstream primer SEQ ID NO:6 were used for sequencing the amplified products, fragment A of the human  $I_{1B}$ -adrenergic receptor gene and fragment A of  $\beta_2$ -adrenergic receptor gene, respectively. As shown in Figure 5 and 6, each sequencing read approximately  
25 550 bases. Other primers described herein including primer SEQ ID NOS: 1, 2, 3, 5, and 7 can also be used for direct sequencing with high reliability. By use of an automated sequencer and sequencing PCR products from in excess of 15 different subjects, we obtained consistent results in accordance with the published coding sequences of the human  $\beta_2$ - and exon 1 of the human  $I_{1B}$ -adrenergic receptor.

Repeated sequencing of PCR products of the same individuals revealed a 100% reliability of our PCR methods without requirement for repeat isolation of PCR fragments. Occasionally occurring "ambiguous reads", which are the result of a reading error of the automated sequencer, can generally be corrected afterwards without re-isolating and sequencing the PCR fragments (Figures 5 and 6).

#### Example 6

#### Use of $\alpha_{1B}$ -AR or $\beta_2$ -AR primer sets for diagnosing diseases associated with genetic alterations in $\alpha_{1B}$ -AR and $\beta_2$ -AR

$\alpha_{1B}$ -AR or  $\beta_2$ -AR primer sets are used to amplify large fragments of the receptor genes, which can subsequently be employed in sequence analyses to detect, prognose, diagnose, or monitor various disease conditions based on sequence variations identified in the amplified fragments. Disease conditions resulting from genetic alterations of  $\alpha_{1B}$ -AR and  $\beta_2$ -AR can include pulmonary, endocrine-metabolic, neuronal, and cardiovascular disorders. For example, the  $\alpha_{1B}$ -AR or  $\beta_2$ -AR gene of subject X is amplified using the primer sets disclosed herein, by a method such as PCR. The resultant gene fragments are analyzed by at least one of the sequence analytical steps, preferably by e.g. direct nucleotide sequencing. By comparing the nucleotide sequence of the receptor gene of subject X to the wild-type receptor sequence, the occurrence of a mutation and/or polymorphism is determined. Further sequence comparison with a positive control, that carries the same variant receptor gene and exhibits syndromes characteristic of the disease of interest, establishes a positive correlation between the two, which indicates the presence or a predisposition of said disease in subject X. Additional sequence comparison with a negative control that retains the wild-type receptor gene and lacks the clinical syndromes confirms the diagnosis.

Table 1.

<b>L<sub>1B</sub>-adrenergic receptor</b>		<b>Primer-sequence</b>	
<b>Primer set no. 1</b>	Upstream (SEQ ID NO:1)	5'-CGG GGG AAG CAA AGT TTC A-3'	
	Downstream (SEQ ID NO:2)	5'-CGG CAG TAC ATG ACT AGA AT-3'	
<b>Primer set no. 2</b>	Upstream (SEQ ID NO:3)	5'-CTC TCC TTG GGT GGA AGG A-3'	
	Downstream (SEQ ID NO:4)	5'-AGC TCA TCA GTA AAC CCA AG-3'	
<b>β<sub>1</sub>-adrenergic receptor</b>		<b>Primer-Sequence</b>	
<b>Primer set no. 1</b>	Upstream (SEQ ID NO:5)	5'-GAA TGA GGC TTC CAG GCG TC-3'	
	Downstream (SEQ ID NO:6)	5'-GAT GAT GCC TAA CGT CTT G-3'	
<b>Primer set no. 2</b>	Upstream (SEQ ID NO:7)	5'-TTC TAC GTG CCC CTG GTG-3'	
	Downstream (SEQ ID NO:8)	5'-TCC TCT AGG ACT AAA GCT C-3'	

Claims

1. An oligonucleotide primer pair for amplifying a human  $\alpha_{1B}$ -adrenergic receptor gene, wherein each individual primer is non-self hybridizing, contains at least 15 nucleotides, has a melting temperature within the range of 50°C to 85°C; wherein said primer pair is non-cross hybridizing, anneals to two distinct regions, which are separated by a distance of at least about 400 nucleotides; wherein said primer pair has the property of yielding a substantially homogenous plurality of gene segments flanked by said regions in a polymerase chain reaction; and wherein at least one primer of the pair has the property of extending 3' end sequence complementary to a template sequence in a DNA polymerase reaction.
2. An oligonucleotide primer pair of claim 1, wherein said primer pair amplifies a fragment selected from the group consisting of: region A in Figure 1; and region B in Figure 1.
3. An oligonucleotide primer pair of claim 2, wherein each individual primer of said pair comprises a linear sequence essentially identical to the polynucleotide shown in SEQ ID No:1 5'CGGGGGAAGCAAAGTTTCA3' or SEQ ID No:2 5'CGGCAGTACATGACTAGAAT3'.
4. An oligonucleotide primer pair of claim 2, wherein at least one primer of said pair comprises a linear sequence essentially identical to the polynucleotide shown in SEQ ID No:1 5'CGGGGGAAGCAAAGTTTCA3' or SEQ ID No:2 5'CGGCAGTACATGACTAGAAT3'.
5. An oligonucleotide primer pair of claim 2, wherein each individual primer of said pair comprises a linear sequence essentially identical to the

polynucleotide shown in SEQ ID No:3 5'CTCTCCTTGGGTGGAAGGA3' or SEQ ID No:4 5'AGCTCATCAGTAAACCCAAG3'.

5 6. An oligonucleotide primer pair of claim 2, wherein at least one primer of said pair comprises a linear sequence essentially identical to the polynucleotide shown in SEQ ID No:3 5'CTCTCCTTGGGTGGAAGGA3' or SEQ ID No:4 5'AGCTCATCAGTAAACCCAAG3'.

10 7. An oligonucleotide primer pair of claim 2 having the nucleotide sequences SEQ ID No:1 5'CGGGGGAAGCAAAGTTTCA3' and SEQ ID No:2 5'CGGCAGTACATGACTAGAAT3'.

15 8. An oligonucleotide primer pair of claim 2, wherein at least one primer of said pair has the nucleotide sequence SEQ ID No:1 5'CGGGGGAAGCAAAGTTTCA3' or SEQ ID No:2 5'CGGCAGTACATGACTAGAAT3'.

20 9. An oligonucleotide primer pair of claim 2 having the nucleotide sequences SEQ ID No:3 5'CTCTCCTTGGGTGGAAGGA3' and SEQ ID No:4 5'AGCTCATCAGTAAACCCAAG3'.

25 10. An oligonucleotide primer pair of claim 2, wherein at least one primer of said pair has the nucleotide sequence SEQ ID No:3 5'CTCTCCTTGGGTGGAAGGA3' or SEQ ID No:4 5'AGCTCATCAGTAAACCCAAG3'.

11. An oligonucleotide primer pair for amplifying a human  $\beta_2$ -adrenergic receptor gene, wherein each individual primer is non-self hybridizing, contains at least 15 nucleotides, has a melting temperature within the range of 50°C to 85°C; wherein said primer pair is non-cross hybridizing, anneals to two distinct regions, which are separated by a distance of at least about 400 nucleotides; wherein said primer pair has the property of yielding a substantially homogenous plurality of gene segments flanked by said regions in a polymerase chain reaction; and wherein at least one primer of the pair has the property of extending 3' end sequence complementary to a template sequence in a DNA polymerase reaction.

12. An oligonucleotide primer pair of claim 11, wherein said primer pair amplifies a fragment selected from the group consisting of: region A in Figure 2; and region B in Figure 2.

13. An oligonucleotide primer pair of claim 12, wherein each individual primer of said pair comprises a linear sequence essentially identical to the polynucleotide shown in SEQ ID No:5 5'GAATGAGGCTTCCAGGCGTC3' or SEQ ID No:6 5'GATGATGCCTAACGTCTTG3'.

14. An oligonucleotide primer pair of claim 12, wherein at least one primer of said pair comprises a linear sequence essentially identical to the polynucleotide shown in SEQ ID No:5 5'GAATGAGGCTTCCAGGCGTC3' or SEQ ID No:6 5'GATGATGCCTAACGTCTTG3'.

15. An oligonucleotide primer pair of claim 12, wherein each individual primer of said pair comprises a linear sequence essentially identical to the

polynucleotide shown in SEQ ID No:7 5'TTCTACGTGCCCCTGGTG3' or SEQ ID No:8 5'TCCTCTAGGACTAAAGCTC3'.

16. An oligonucleotide primer pair of claim 12, wherein at least one  
5 primer of said pair comprises a linear sequence essentially identical to the  
polynucleotide shown in SEQ ID No:7 5'TTCTACGTGCCCCTGGTG3' or SEQ ID  
No:8 5'TCCTCTAGGACTAAAGCTC3'.

17. An oligonucleotide primer pair of claim 12 having the nucleotide  
10 sequences SEQ ID No:5 5'GAATGAGGCTTCCAGGCGTC3' and SEQ ID No:6  
5'GATGATGCCTAACGTCTTG3'.

18. An oligonucleotide primer pair of claim 12, wherein at least one  
15 primer of said pair has the nucleotide sequence SEQ ID No:5  
5'GAATGAGGCTTCCAGGCGTC3' or SEQ ID No:6  
5'GATGATGCCTAACGTCTTG3'.

19. An oligonucleotide primer pair of claim 12 having the nucleotide  
20 sequences SEQ ID No:7 5'TTCTACGTGCCCCTGGTG3' and SEQ ID No:8  
5'TCCTCTAGGACTAAAGCTC3'.

20. An oligonucleotide primer pair of claim 12, wherein at least one  
primer of said pair has the nucleotide sequence SEQ ID No:7  
5'TTCTACGTGCCCCTGGTG3' or SEQ ID No:8  
25 5'TCCTCTAGGACTAAAGCTC3'.



21. A method of amplifying a segment of a human  $I_{IB}$ -adrenergic receptor gene of a subject comprising the steps of:

a) providing a biological sample of the subject containing nucleic acid molecules;

b) amplifying a segment of the gene by employing an oligonucleotide primer pair of claim 1, a primer-dependent DNA polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of segments of the gene.

22. A method for identifying a genetic variation in a human  $I_{IB}$ -adrenergic receptor gene of a subject comprising the steps of:

a) providing a biological sample of the subject containing nucleic acid molecules;

b) amplifying a segment of the  $I_{IB}$ -adrenergic receptor gene by employing an oligonucleotide primer pair of claim 1, a primer-dependent DNA polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of amplified segments of the gene; and

c) identifying a sequence variation of the resulting amplified products relative to a control using at least one sequence analytical step.

23. A method for identifying a genetic variation in a human  $I_{IB}$ -adrenergic receptor gene of a subject according to claim 22, wherein the sequence analytical step is selected from the group of nucleotide sequencing, single-strand conformation polymorphism assay, allele-specific oligonucleotide hybridization, Southern blot analysis, and restriction endonuclease digestion.

24. A method for diagnosing a disease associated with a genetic alteration of a human  $\alpha_{1B}$ -adrenergic receptor gene of a subject comprising the step of:

a) providing a biological sample of the subject containing nucleic acid molecules;

5 b) amplifying a segment of the  $\alpha_{1B}$ -adrenergic receptor gene by employing an oligonucleotide primer pair of claim 1, a primer-dependent DNA polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of amplified segments of the gene;

10 c) identifying a sequence variation of the resulting amplified products relative to a control using at least one sequence analytical step; and

d) determining a correlation of the detected variation between the subject and a control.

15 25. A method for diagnosing a disease associated with a genetic alteration of a human  $\alpha_{1B}$ -adrenergic receptor of a subject of claim 24, wherein the sequence analytical step is selected from the group of nucleotide sequencing, single-strand conformation polymorphism assay, allele-specific oligonucleotide hybridization, Southern blot analysis, and restriction endonuclease digestion.

20 26. A method of amplifying a segment of a human  $\beta_2$ -adrenergic receptor gene of a subject comprising the steps of:

a) providing a biological sample of the subject containing nucleic acid molecules;

25 b) amplifying a segment of the gene by employing an oligonucleotide primer pair of claim 11, a primer-dependent DNA polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of segments of the gene.

27. A method for identifying a genetic variation in a human  $\beta_2$ -adrenergic receptor gene of a subject comprising the steps of:

5 a) providing a biological sample of the subject containing nucleic acid molecules;

b) amplifying a segment of the gene by employing an oligonucleotide primer pair of claim 11, a primer-dependent DNA polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of segments of the gene; and

10 c) identifying a sequence variation of the resulting amplified products relative to a control using at least one sequence analytical step.

28. A method for identifying a genetic variation in a human  $\beta_2$ -adrenergic receptor gene of a subject according to claim 27, wherein the sequence analytical step  
15 is selected from the group of nucleotide sequencing, single-strand conformation polymorphism assay, allele-specific oligonucleotide hybridization, Southern blot analysis, and restriction endonuclease digestion.

29. A method for diagnosing a disease associated with a genetic alteration  
20 of a human  $\beta_2$ -adrenergic receptor gene of a subject comprising the step of:

a) providing a biological sample of the subject containing nucleic acid molecules;

b) amplifying a segment of the gene encoding said receptor by  
25 employing an oligonucleotide primer pair of claim 11, a primer-dependent DNA polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of amplified segments of the gene;

- c) identifying a sequence variation of the resulting amplified products relative to a control using at least one sequence analytical step; and
- d) determining a correlation of the detected variation between the subject and a control.

5

30. A method for diagnosing a disease associated with a genetic alteration of a human  $\beta_2$ -adrenergic receptor gene of a subject of claim 29, wherein the sequence analytical step is selected from the group of nucleotide sequencing, single-strand conformation polymorphism assay, allele-specific oligonucleotide hybridization, Southern blot analysis, and restriction endonuclease digestion.

10

31. A method for diagnosing a disease associated with a genetic alteration of a human  $\alpha_{1B}$ -adrenergic receptor gene of a subject of claim 24, wherein said disease is a cardiovascular disorder.

15

32. A method for diagnosing a disease associated with a genetic alteration of a human  $\alpha_{1B}$ -adrenergic receptor gene of a subject of claim 24, wherein said disease is hypertension.

20

33. A method for diagnosing a disease associated with a genetic alteration of a human  $\alpha_{1B}$ -adrenergic receptor gene of a subject of claim 24, wherein said disease is a prostatic disorder.

25

34. A method for diagnosing a disease associated with a genetic alteration of a human  $\beta_2$ -adrenergic receptor gene of a subject of claim 29, wherein said disease is a cardiovascular disorder.

35. A method for diagnosing a disease associated with a genetic alteration of a human  $\beta_2$ -adrenergic receptor gene of a subject of claim 29, wherein said disease is hypertension.

36. A method for diagnosing a disease associated with a genetic alteration of a human  $\beta_2$ -adrenergic receptor gene of a subject of claim 29, wherein said disease is asthma.

10

Figure 1

ctccgaggaat tctcttttccc tcttttaggt gctgggacaag ttgcataatcc 50  
 cgttaaatcc gggggcccaact cccggagccc cctatttaaag gtaagcagcc 100  
 cccaaaccac cttttgacacg gaagagtgac tagcggggaa aataaaccaca 150  
 gcggccgcct aggggtgggt gacccgcgat ctccacgccc aggtcccgcc 200  
 ctcccgcccc ttcccccgtc cccgcccccc gttagagtgga cctcgggagc 250  
 ggccggggag gttggcttctc ggtcttgccg gtagtgaccc ccgatctcca 300  
 cggccaaggt ccgcccctcgc ggeccctgcc cccgcccccc cccgcaagag 350  
 gcgcgcccctg ggagcgccgg ggtaacgcgg ctggcttccc cgttcgccc 400  
 gtggcgccgt cggggctcgc ggcgtccttg gctggacccg cattgcccc 450  
 tagtcgcgcg cggagtcagg gcgcggggct cccccgcctg atgtcacccc 500  
 cgtgcagtcg gcccagaagc ggctcattga agcagaccc ctctggcgct 550  
 cgtcggggcg aggagccgccc ccccccctct cccctccgct ccccgccgag 600  
 cgcggggcgc ccccgccctcc ccccccctct cccctccgct ccccgccgag 650  
 cccggccagg cgcgctcctg acgtggacca ttaaaccttg agctgcccgc 700  
 tcgtccctct tccctcctct cctccctctg acagccgagc gacgcactcg 750  
 gtgcaggcag gagacgtgct gcgggctggg ctgccccggg cagatgactc 800  
 ctgccaggag gggcgccctct gggaaagaaga ccacggggga agcaaaagttc 850  
 gggggcagct gaggagccctt cgcgcgagcc ctcccgagcc caatcattccc 900  
 cctggcctatg gaggcgccgac tctaaagatga ATCCCGACCT GGACACCGGC 950  
 CACATACAT CAGCACCTGC CCACTGGGGA GAGTTGAAA ATGCCAATCT 1000  
 CACTGGCCCC AACACAGCCT CGAGGGGGGG CACACTGCCC CAGCTGGACA 1050  
 TCACCAAGGC CATCTCTGTG GGCCTGGTGC TGGGCGCCTT CATCCTCTTT 1100  
 GCCATCGTGG GCAACATCTT AGTCATCTTG TCTGTGGCCT GCAACCGGCA 1150  
 CCTGCGGACG CCAACCACT ACTTCATTGT CAACCTGGCC ATGGCCGACC 1200  
 TGCTGTTGAG CTTACCCGTC CTGCCCTTCT CAGCGGCCCT AGAGGTGCTC 1250  
 GGCTACTGGG TGCTGGGGCG GATCTTCTGT GACATCTGGG CAGCCGTGSA 1300  
 TGTCCTGTGC TGCCACAGCT CCATTCTGAG CCTGTGCGCC ATCTCCATCG 1350  
 ATCGCTACAT GGGGGTGGCG TACTCTCTGC AGTATCCAC GCTGGTCAAC 1400  
 CCGAGGAAAG CCACTCTTGGC GCTGCTCAGT GTCTGGGTCT TGTCCACCGT 1450  
 CATCTCCATC GGGCTCTICC TTGGGTGGA GGGGCCGGCA CCCAACGATG 1500  
 ACAAGGAGTG CCGGCTCACC GAAGAACCTT TCTATGCCCT CTTCTCTCT 1550  
 CTGGGCTCCT TCTACATCCC TCTGGCGGTC ATTCTAGTCA TGTACTGCCG 1600  
 TGTCTATATA GTGGCCAAAG GAACCAACAA GAACCTAGAG GCAGGAGTCA 1650  
 TGAAGGAGAT TGCCAACTCC AAGGAGCTCA CCTGAGGAT CCATTCCAA 1700  
 AACTTTTCAG AGGACACCTT TAGCAGTACC AAGGCCAAGG GCCACAACCC 1750  
 CAGGAGTTCC ATAGCTGTCA AACTTTTFAA GTTCTCCAGG GAAAAGAAAG 1800  
 CAGCTAAGAC GTTGGGCAAT GTGGTCGGTA TGTTCATCTT GTGCTGGCTA 1850  
 CCCTTCTTCA TCGCTCTACC GCTGTGtaag ttggccacta gcagcagggg 1900  
 gactggggcat ttttggagct tgggtttact catcagctta ctctaaagt 1950  
 ttttgtgggt tttgtttctt atgcagctcg tgcgtgttcg gagattgaa 2000  
 aatattgttt gttctgcaaa gggttttgag attggggagc tggctaaaaa 2050  
 ccsactcagg tgttagtaga acacgctaag gcactagctt ctgaaataga 2100  
 accagggag gaaaaatctg tatgaggaa gactcactca acagcctcg 2150  
 ttttaataatt aaaaagatat tcactgggct tgaatatcac accggcgta 2200  
 tttcagtagt aatgatgtgt cggctaaggc agcgtcacta atgcagcata 2250  
 caaataagtt tgtagttact gcagagacag catttgga gacagggaagg 2300  
 agctcgtaca cagaaaggca gcattcattc agcatttcca gggctaaggc 2350  
 agaagccacg cttcttcaag agctttcaga ggcctgcatc tctccctccc 2400  
 ctcttcactg acagctatca cccagatata ctacgttcat tatccgtaaa 2450  
 taagagcatt ttacagccac acagcccca ccagccttta agttattgaa 2500  
 agcttgagat gtgaaagaga agatgactag aagggtcacac atttttagtt 2550  
 tttaccccaa aaatgtttct aactctagt atttatgga tggccagiat 2600  
 caaattatag ttgtccctca tctcagctga cagagacca atcctggtaa 2650  
 gatggaattc gggtaaccc

noncoding sequence

SEQ No. 1

SEQ No. 3

SEQ No. 2

SEQ No. 4

A

B

Intron



A. ——— = 1000 bases  
B. ——— = 100 bases



Figure 4

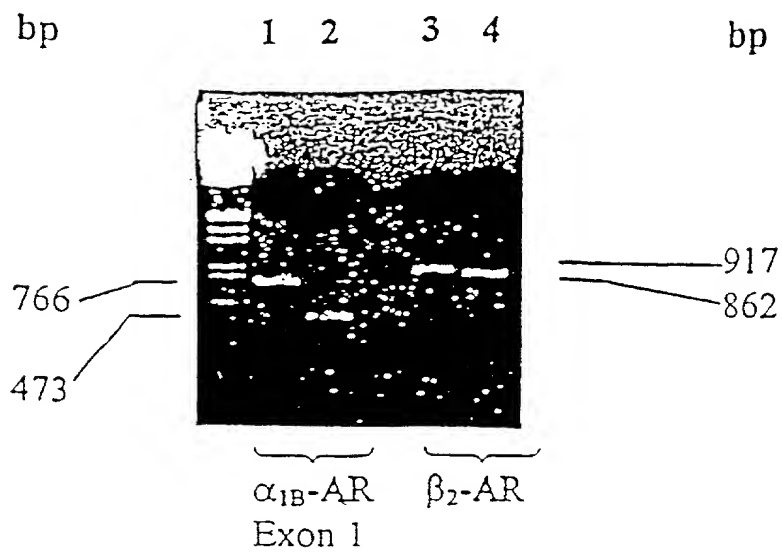


Figure 5

Signal G 272 A:733 T:406 C:502  
DT4%AC/A Set-Amy/Primary  
95091656mavx  
Points 1063 to 11512 Base 1: 1063

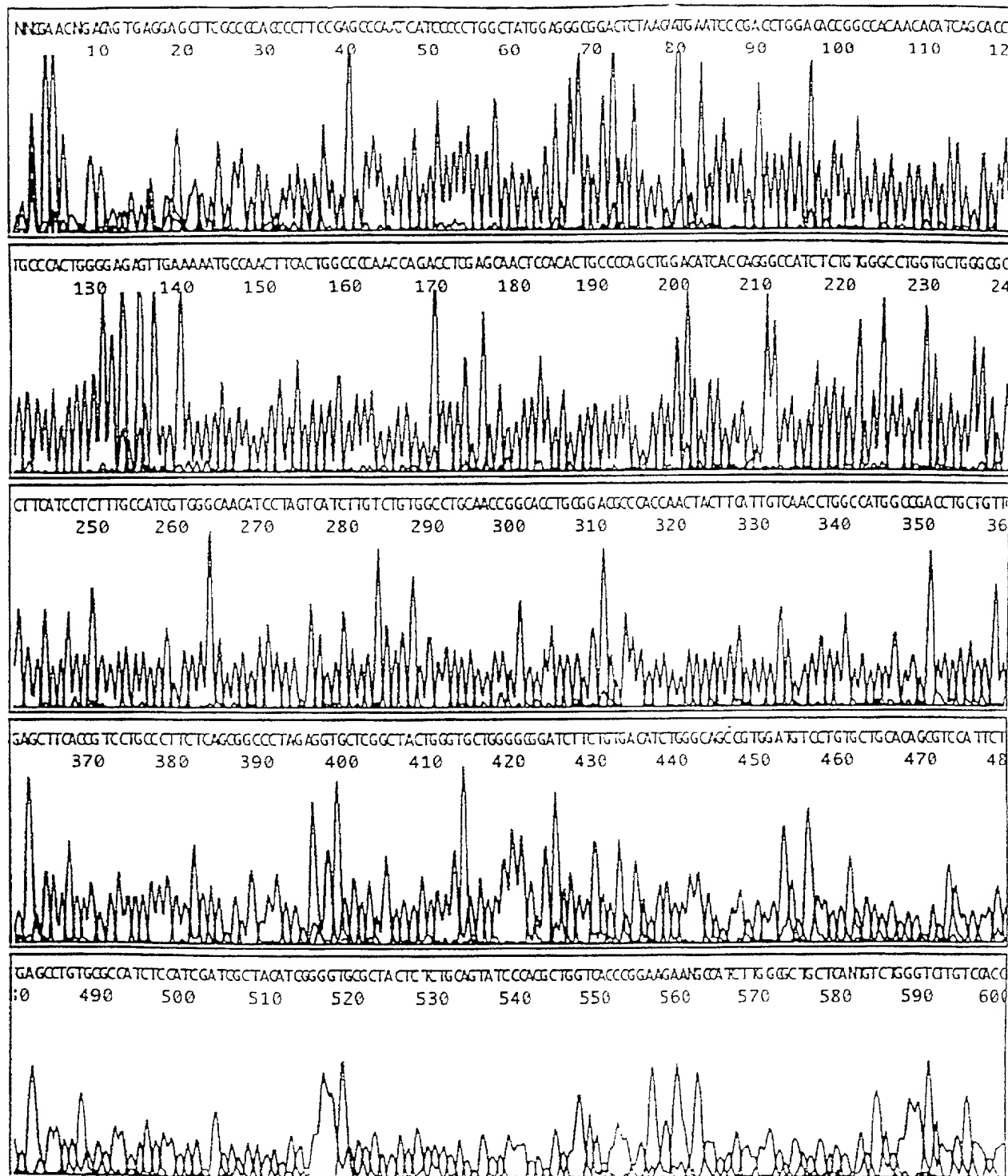


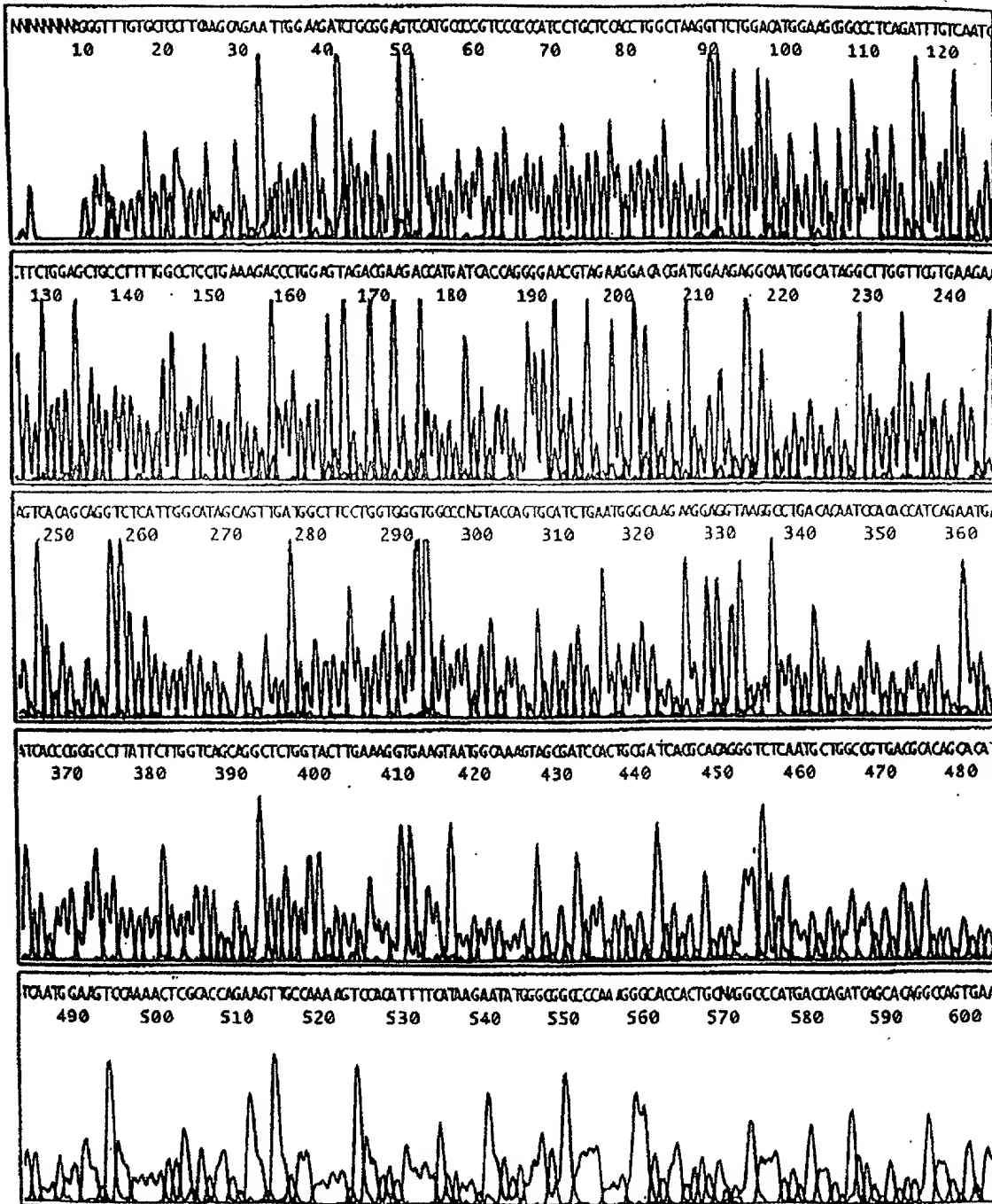
Figure 6

Signal G:358 A:585 T:150 C:163

DT4%Ac(A Set-AnyPrimer)

211 fs laq

Points 1112 to 8444 Base 1: 1112



## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANT: Insel, Paul A.  
Herrmann, Volker  
Buescher, Rainer
- (ii) TITLE OF THE INVENTION: METHODS AND COMPOSITIONS FOR  
IDENTIFYING GENETIC VARIATIONS IN HUMAN alpha-1B AND  
beta-2-ADRENERGIC RECEPTORS
- (iii) NUMBER OF SEQUENCES: 10
- (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: MORRISON & FOERSTER  
(B) STREET: 755 PAGE MILL ROAD  
(C) CITY: PALO ALTO  
(D) STATE: CA  
(E) COUNTRY: USA  
(F) ZIP: 94304-1018
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: DOS  
(D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: Dylan, Tyler M  
(B) REGISTRATION NUMBER: 37,612  
(C) REFERENCE/DOCKET NUMBER: 22000-20589.00
- (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: 650-813-5600  
(B) TELEFAX: 650-494-0792  
(C) TELEX: 706141

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGGGGAAGC AAAGTTTCA

19

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGGCAGTACA TGACTAGAAT

20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTCTCCTTGG GTGGAAGGA

19

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGCTCATCAG TAAACCCAAG

20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAATGAGGCT TCCAGGCGTC

20

(2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATGATGCCT AACGTCTTG

19

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTCTACGTGC CCCTGGTG

18

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCCTCTAGGA CTAAAGCTC

19

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2719 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTCGAGGAAT	TCTCTTTCCC	TCTTTTAGGT	GCTGGACAAG	TTGCATATCC	CGTTAAATCC	60
GGGGCCCACT	CCGACAGCCC	CCTATTAAAG	GTAAGCAGCC	CCCAACCCAC	CTTTGACACG	120
GAAGAGTGAC	TAGCGGGGAA	AATAACCCCA	GCGGCCGCCT	AGGGGTGGGT	GACCCGCGAT	180
CTCCACGCCC	AGGTCCCGCC	CTCCCGCCCC	TTCCCCCGTC	CCCGCCCCCC	GTAGAGGTGA	240
CCCTGGGAGC	GGCGGGGGAG	GTTGGCTTTC	GGTCTTGCGG	GTGGTGACCC	GCGATCTCCA	300
CGCCCCAAGT	CCGCCCTCGC	GGCCCCTGCC	CCCGCCCCCC	CCCGCAAGAG	GCGCGCCCTG	360
GGAGCGGCGG	GGTAACGCGG	CTGGCTTCCC	GGCTTGCGGG	GTGGCGGCGT	CGGGGCTGCG	420
GGCGTCCTTG	GCTGGACCCG	CATTGCCCCC	TAGTGCCGCG	CGGAGTCAGG	GCGCCGGGCT	480
CCCCCGCCTG	ATGTCACCGC	CGTGCAGTCA	GCCAGAAGC	GGCTCATTGA	AGCAGACCCT	540

CTTCGGCGCT CGCTGGGCGG AGGAGCCGCC GCGGTCCGCA GACCCGAGCG AGCTGGGCAC 600  
CGCCGGGCGC CCCCgcctcc CCCCCTCCT CCCCTCCGCT CCCCgCGAG CCCGGCCAGG 660  
CGCGCTCCTG ACGTGGACCA TTAACCTTGG AGCTGCCGCC TCGTCCCCTC TCCTCCTCCT 720  
CCTCCCTCTG ACAGGCGAGC GAGCGACTCG GTGCAGGCAG GAGACGTGCT GCGGGCTGGG 780  
CTGCCCCGGG GAGATGACTC CTGCCAGGAG GGGCGCCTCT GGGAGAAGA CCACGGGGGA 840  
AGCAAAGTTT CAGGGCAGCT GAGGAGCCTT CGCCGCAGCC CTTCCGAGCC CAATCATCCC 900  
CCTGGCTATG GAGGGCGGAC TCTAAGATGA ATCCCGACCT GGACACCGGC CACAACACAT 960  
CAGCACCTGC CCACTGGGGA GAGTTGAAAA ATGCCAACTT CACTGGCCCC AACCAGACCT 1020  
CGAGGGGGGG CACACTGCCC CAGCTGGACA TCACCAGGGC CATCTCTGTG GGCCTGGTGC 1080  
TGGGCGCCTT CATCCTCTTT GCCATCGTGG GCAACATCCT AGTCATCTTG TCTGTGGCCT 1140  
GCAACCGGCA CCTGCGGACG CCCACCAACT ACTTCATTGT CAACCTGGCC ATGGCCGACC 1200  
TGCTGTTGAG CTTACCGTC CTGCCCTTCG CAGCGGCCCT AGAGGTGCTC GGCTACTGGG 1260  
TGCTGGGGCG GATCTTCTGT GACATCTGGG CAGCCGTGGA TGTCTGTGC TGCACAGCGT 1320  
CCATTCTGAG CCTGTGCGCC ATCTCCATCG ATCGCTACAT CGGGGTGCGC TACTCTCTGC 1380  
AGTATCCAC GCTGGTCACC CGGAGGAAGG CCATCTTGGC GCTGCTCAGT GTCTGGGTCT 1440  
TGTCCACCGT CATCTCCATC GGGCCTCTCC TTGGGTGGAA GGAGCCGGCA CCCAACGATG 1500  
ACAAGGAGTG CGGGGTACC GAAGAACCCT TCTATGCCCT CTTCTCCTCT CTGGGCTCCT 1560  
TCTACATCCC TCTGGCGGTG ATTCTAGTCA TGTACTGCCG TGTCTATATA GTGGCCAAGA 1620  
GAACCACCAA GAACCTAGAG GCAGGAGTCA TGAAGGAGAT GTCCAACTCC AAGGAGCTGA 1680  
CCCTGAGGAT CCATTCCAAG AACTTTCACG GTCCAACTCC AAGGAGCTGA CCCTGAGGAT 1740  
CCATTCCAAG AACTTTCACG AGGACACCTT TAGCAGTACC AAGGCCAAGG GCCACAACCC 1800  
CAGGAGTTCC ATAGCTGTCA AACTTTTAA GTTCTCCAGG GAAAAGAAAG CAGCTAAGAC 1860  
GTTGGGCATT GTGGTCGGTA TGTTTCATCT TGTCTGGCTA CCCTTCTTCA TCGCTCTACC 1920  
GCTTGTTAAG TTGGGCACTA GCAGCAGGGG GACTGGGCAT TTTTGGACCT TGGGTTTACT 1980  
GATGAGCTTA CTCTAAAGTT TTTTGTGGGT TTTGTTTCTT ATGCAGTCTG TGCCTGTTCG 2040  
GAGATTGAAT AATATTGTTT GTTCTGCAAA GGGTTTGAG ATTGGGGAGC TGGCTAAAAA 2100  
CCAATCAGG TGTTAGTAGA ACACGCTAAG GCACTAGCTT CTGAAATAGA ACCAGGGAAG 2160  
GAAAATCTGG TATGAGGAAT GACTCACTCA ACAGCCTCGG TTTAATAATT AAAAGATAT 2220  
TCACTGGGCT TGAATATCAC ACCGGCGTTA TTTAGTAGT AATGATGTGT CGGCTAAGGC 2280  
AGCGTCACTA ATGCAGCATA CAAAATAGTT TGTAAGTACT GCAGAGACAG CATTTGGGAA 2340  
GCAGGGAGGC AGCTCGTACA CAGAAAGGCA GCATTCAATC AGCATTTCCA GGGCTAGAGC 2400  
AGAAGCCACG CTTCTTCAAG AGCTTTCAGA GGCTGCATT TCTCCCTCCC CTCTTCACTG 2460  
ACAGCTATCA CCCAGATATA CTACGTTTAT TATCCGTTAA TAAGAGCATT TTACAGCCAC 2520  
ACAGCCCCAA CCAGCCTTTA AGTTATTGAA ATCTTGAGAT GTGAAAGAGA AGATGACTAG 2580  
AGGGTCACAC ATTTTATAGT TTTACCCCAA AAATGTTTCT AACTCTAGTG ATTTATGGAA 2640  
TGCCAGAAAT CAAATTATAG TTGTCCCTCA TCTCAGCTGA CAGAGACCAA ATCCTGGTAA 2700  
GATGGAATTC GGGTACCCC 2719

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2300 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAATTCATGC CGCGTTTCTG TGTGGGACAG GGGTGACTTT GTGCCGGATG GCTTCTGTGT 60  
GAGAGCCGCG GCGAGTGTGC ATGTCGGTGA GCTGGGAGGG TGTGTCTCAG TGTCTATGGC 120  
TGTGGTTCGG TATAAGTCTA AGCATGTCTG CCAGGGTGTA TTTGTGCCTG TATGTGCGTG 180  
CCTCGGTGGG CACTCTCGTT TCCTTCCGAA TGTGGGGCAG TGCCGGTGTG CTGCCCTCTG 240  
CCTTGAGACC TCAAGCCGCG CAGGCGCCCA GGGCAGGCAG GTAGCGGCCA CAGAAGAGCC 300  
AAAAGCTCCC GGGTTGGCTG GTAAGCACAC CACCTCCAGC TTTAGCCCTC TGGGGCCAGC 360

CAGGGTAGCC GGGGAAGCAGT GGTGGCCCCG CCTCCAGGGA GCAGTTGGGC CCCGCCCCGG 420  
 CCAGCCTCAG GAGAAGGAGG GCGAGGGGAG GGGAGGGAAA GGGGAGGAGT GCCTCGCCCC 480  
 TTCGCGGGCTG CCGGCGTGCC APTGGCCGAA AGTTCCCGTA CGTCACGGCG AGGGCAGTTC 540  
 CCCTAAAGTC CTGTGCACAT AACGGGCAGA ACGCACTGCG AAGCGGCTTC TTCAGAGCAC 600  
 GGGCTGGAAC TGGCAGGCAC CGCGAGCCCC TAGTACCCGA CAAGCTGAGT GTGCAGGACG 660  
 AGTCCCCACC ACACCCACAC CACAGCCGCT AAATGAGGCT TCCAGGCGTC CGCTCGCGGC 720  
 CCGCAGAGCC CCGCCGTGGG TCCGCCTGCT GAGGCGCCCC CAGCCAGTGC GCTTACCTGC 780  
 CAGACTGCGT GCCATGGGGC AACC CGGGAA CGGCAGCGCC TTCTTGCTGG CACCCAATAG 840  
 AAGCCATGCG CCGGACCACG ACCTCACGCA GCAAAGGGAC GAGGTGTGGG TGGTGGGCAT 900  
 GGGCATCGTC ATGTCTCTCA TCGTCCTGGC CATCGTGTTT GGCAATGTGC TGGTCATCAC 960  
 AGCCATTGCC AAGTTCGAGC GTCTGCAGAC GGTCAACCAAC TACTTCATCA CTTCACTGGC 1020  
 CTGTGCTGAT CTGGTCATGG GCCTGGCAGT GGTGCCCTTT GGGGCCGCCC ATATTCTTAT 1080  
 GAAAATGTGG ACTTTTGGCA ACTTCTGGTG GCAGTTTGG ACTTCCATTG ATGTGCTGTG 1140  
 CGTCACGGCC AGCATTGAGA CCCTGTGCGT GATCGCAGTG GATCGCTACT TTGCCATTAC 1200  
 TTCACCTTTC AAGTACCAGA GCCTGCTGAC CAAGAATAAG GCGCGGGTGA TCATTCTGAT 1260  
 GGTGTGGATT GTGT CAGGCC TTACCTCCTT CTGCCCCATT CAGATGCACT GGTACCGGGC 1320  
 CACCCACCAG GAAGCCATCA ACTGCTATGC CAATGAGACC TGCTGTGACT TCTTCACGAA 1380  
 CCAAGCCTAT GCCATTGCCT CTTCCATCGT GTCCTTCTAC GTTCCCCTGG TGATCATGGT 1440  
 CTTCTGCTAC TCCAGGGTCT TTCAGGAGGC CAAAAGGCAG CTCCAGAAGA TTGACAAATC 1500  
 TGAGGGCCGC TTCCATGTCC AGAACCTTAG CCAGTGAGG CAGGATGGGC GGACGGGGCA 1560  
 TGGACTCCGC AGATCTTCCA AGTTCCTGCTT GAAGGAGCAC AAAGCCCTCA AGACGTTAGG 1620  
 CATCATCATG GGCACCTTCA CCCTCTGCTG GCTGCCCTTC TTCATCGTTA ACATTGTGCA 1680  
 TGTGATCCAG GATAACCTCA TCCGTAAGGA AGTTTACATC CTCTAAATT GGATAGGCTA 1740  
 TGTCAATTCT GGTTCATC CCCTTATCTA CTGCCGGAGC CCAGATTTCA GGATTGCCTT 1800  
 CCAGGAGCTT CTGTGCCTGC GCAGGTCTTC TTTGAAGGCC TATGGGAATG GCTACTCCAG 1860  
 CAACGGCAAC ACAGGGGAGC AGAGTGGATA TCACGTGGAA CAGGAGAAAG AAAATAAACT 1920  
 GCTGTGTGAA GACCTCCCAG GCACGGAAGA CTTTGTGGGC CATCAAGGTA CTGTGCCTAG 1980  
 CGATAACATT GATTACAAG GGAGGAATTG TAGTACAAAT GACTCACTGC TGTAAGCAG 2040  
 TTTTCTACT TTTAAAGACC CCCCCCCCCA ACAGAACACT AAACAGACTA TTTAACTTGA 2100  
 GGGTAATAAA CTTAGAATAA AATTGTAAAA TTGTATAGAG ATATGCAGAA GGAAGGGCAT 2160  
 CCTTCTGCCT TTTTATTTT TTTAAGCTGT AAAAAGAGAG AAAACTTATT TGAGTGATTA 2220  
 TTTGTTATTT GTACAGTTCA GTTCCTCTTT GCATGGAATT TGTAAGTTTA TGTCTAAAGA 2280  
 GCTTTAGTCC TAGAGGACCT



**DECLARATION FOR PATENT APPLICATION**  
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER  
220002058901

As a below named inventors we hereby declare that:

Our residence, post office address and citizenship are as stated below next to our name,

We believe we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**METHODS AND COMPOSITIONS FOR IDENTIFYING VARIATIONS IN HUMAN  $\alpha_{1B}$  AND  $\beta_2$ -ADRENERGIC RECEPTOR GENES**

the specification of which (check only one item below):

- ☐ is attached hereto.
- ☒ was filed as United States application  
Serial No. 09/402,224  
on September 29, 1999  
and was amended on (if applicable).
- ☐ was filed as PCT international application  
Number  
on  
and was amended under PCT Article 19  
on (if applicable).

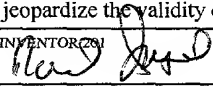
We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37 Code of Federal Regulations § 1.56(a) and (b).

We hereby claim foreign priority benefits under Title 35 United States Code § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

**PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. § 119:**

COUNTRY (if PCT indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. § 119
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

<b>Declaration for Patent Application (Continued)</b> (Includes Reference to PCT International Applications)				ATTORNEY'S DOCKET NUMBER 220002058901	
We hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:					
<b>PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. § 120:</b>					
U.S. APPLICATIONS			STATUS (Check one)		
U S APPLICATION NUMBER	U S FILING DATE	PATENTED	PENDING	ABANDONED	
60/086,232	November 10, 1997			X	
PCT APPLICATIONS DESIGNATING THE U.S.			STATUS (Check one)		
PCT APPLICATION NUMBER	PCT FILING DATE	U S SERIAL NUMBERS ASSIGNED (if any)	PATENTED	PENDING	ABANDONED
PCT/US98/23496	November 4, 1998				X
Send correspondence to: Catherine M. Polizzi Morrison & Foerster LLP 755 Page Mill Road Palo Alto, California 94304-1018				Direct telephone calls to: Catherine M. Polizzi at (650) 813-5651	
201	FULL NAME OF INVENTOR INSEL	FAMILY NAME INSEL	FIRST GIVEN NAME Paul	SECOND GIVEN NAME A.	
	RESIDENCE & CITIZENSHIP La Jolla CA	CITY La Jolla	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP United States	
	POST OFFICE ADDRESS 8660 Kilbourn Drive	CITY La Jolla	STATE & ZIP CODE/COUNTRY California 92037	STATE & ZIP CODE/COUNTRY California 92037	
202	FULL NAME OF INVENTOR HERRMANN	FAMILY NAME HERRMANN	FIRST GIVEN NAME Volker	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP Freiburg DEX	CITY Freiburg	STATE OR FOREIGN COUNTRY Germany	COUNTRY OF CITIZENSHIP Germany	
	POST OFFICE ADDRESS In der Roete 18	CITY 79104 Freiburg	STATE & ZIP CODE/COUNTRY Germany	STATE & ZIP CODE/COUNTRY Germany	
203	FULL NAME OF INVENTOR BUESCHER	FAMILY NAME BUESCHER	FIRST GIVEN NAME Rainer	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP Essen DEX	CITY Essen	STATE OR FOREIGN COUNTRY Germany	COUNTRY OF CITIZENSHIP Germany	
	POST OFFICE ADDRESS Germarkenstraße 84	CITY 45147 Essen	STATE & ZIP CODE/COUNTRY Germany	STATE & ZIP CODE/COUNTRY Germany	
204	FULL NAME OF INVENTOR LIGGETT	FAMILY NAME LIGGETT	FIRST GIVEN NAME Stephen	SECOND GIVEN NAME B.	
	RESIDENCE & CITIZENSHIP Cincinnati	CITY Cincinnati	STATE OR FOREIGN COUNTRY Ohio	COUNTRY OF CITIZENSHIP United States	
	POST OFFICE ADDRESS 8020 Elbrecht Drive	CITY Cincinnati	STATE & ZIP CODE/COUNTRY Ohio 45242	STATE & ZIP CODE/COUNTRY Ohio 45242	
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.					
SIGNATURE OF INVENTOR 201 		SIGNATURE OF INVENTOR 202		SIGNATURE OF INVENTOR 203	
DATE 10/12/00		DATE		DATE	
SIGNATURE OF INVENTOR 204					
DATE					

**DECLARATION FOR PATENT APPLICATION**  
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER  
220002058901

As a below named inventors we hereby declare that:

Our residence, post office address and citizenship are as stated below next to our name,

We believe we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**METHODS AND COMPOSITIONS FOR IDENTIFYING VARIATIONS IN HUMAN  $\alpha_{1B}$ - AND  $\beta_2$ -ADRENERGIC RECEPTOR GENES**

the specification of which (check only one item below):

- ☐ is attached hereto.
- ☒ was filed as United States application  
Serial No. 09/402,224  
on September 29, 1999  
and was amended on (if applicable).
- ☐ was filed as PCT international application  
Number  
on  
and was amended under PCT Article 19  
on (if applicable).

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37 Code of Federal Regulations § 1.56(a) and (b).

We hereby claim foreign priority benefits under Title 35 United States Code § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

**PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. § 119:**

COUNTRY (if PCT indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. § 119
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

<b>Declaration for Patent Application (Continued)</b> (Includes Reference to PCT International Applications)				ATTORNEY'S DOCKET NUMBER 220002058901	
We hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:					
<b>PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. § 120:</b>					
U.S. APPLICATIONS			STATUS (Check one)		
U S APPLICATION NUMBER	U S FILING DATE	PATENTED	PENDING	ABANDONED	
60/086,232	November 10, 1997			X	
PCT APPLICATIONS DESIGNATING THE U.S.			STATUS (Check one)		
PCT APPLICATION NUMBER	PCT FILING DATE	U S SERIAL NUMBERS ASSIGNED (if any)	PATENTED	PENDING	ABANDONED
PCT/US98/23496	November 4, 1998				X
<b>Send correspondence to:</b> Catherine M. Polizzi Morrison & Foerster LLP 755 Page Mill Road Palo Alto, California 94304-1018			<b>Direct telephone calls to:</b> Catherine M. Polizzi at (650) 813-5651		
201	FULL NAME OF INVENTOR INSEL	FAMILY NAME INSEL	FIRST GIVEN NAME Paul	SECOND GIVEN NAME A.	
	RESIDENCE & CITIZENSHIP La Jolla	CITY La Jolla	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP United States	
	POST OFFICE ADDRESS 8660 Kilbourn Drive	POST OFFICE ADDRESS 8660 Kilbourn Drive	CITY La Jolla	STATE & ZIP CODE/COUNTRY California 92037	
202	FULL NAME OF INVENTOR HERRMANN	FAMILY NAME HERRMANN	FIRST GIVEN NAME Volker	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP Freiburg	CITY Freiburg	STATE OR FOREIGN COUNTRY Germany	COUNTRY OF CITIZENSHIP Germany	
	POST OFFICE ADDRESS In der Roete 18	POST OFFICE ADDRESS In der Roete 18	CITY 79104 Freiburg	STATE & ZIP CODE/COUNTRY Germany	
203	FULL NAME OF INVENTOR BUESCHER	FAMILY NAME BUESCHER	FIRST GIVEN NAME Rainer	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP Essen	CITY Essen	STATE OR FOREIGN COUNTRY Germany	COUNTRY OF CITIZENSHIP Germany	
	POST OFFICE ADDRESS Germarkenstraße 84	POST OFFICE ADDRESS Germarkenstraße 84	CITY 45147 Essen	STATE & ZIP CODE/COUNTRY Germany	
204	FULL NAME OF INVENTOR LIGGETT	FAMILY NAME LIGGETT	FIRST GIVEN NAME Stephen	SECOND GIVEN NAME B.	
	RESIDENCE & CITIZENSHIP Cincinnati	CITY Cincinnati	STATE OR FOREIGN COUNTRY Ohio	COUNTRY OF CITIZENSHIP United States	
	POST OFFICE ADDRESS 8020 Elbrecht Drive	POST OFFICE ADDRESS 8020 Elbrecht Drive	CITY Cincinnati	STATE & ZIP CODE/COUNTRY Ohio 45242	
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.					
SIGNATURE OF INVENTOR 201		SIGNATURE OF INVENTOR 202		SIGNATURE OF INVENTOR 203	
DATE		DATE		DATE	
SIGNATURE OF INVENTOR 204					
DATE					

**DECLARATION FOR PATENT APPLICATION**  
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER  
220002058901

As a below named inventors we hereby declare that:

Our residence, post office address and citizenship are as stated below next to our name,

We believe we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**METHODS AND COMPOSITIONS FOR IDENTIFYING VARIATIONS IN HUMAN  $\alpha_{1B}$  AND  $\beta_2$ -ADRENERGIC RECEPTOR GENES**

the specification of which (check only one item below):

- ☐ is attached hereto.
- ☒ was filed as United States application  
Serial No. 09/402,224  
on September 29, 1999  
and was amended on (if applicable).
- ☐ was filed as PCT international application  
Number  
on  
and was amended under PCT Article 19  
on (if applicable).

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37 Code of Federal Regulations § 1.56(a) and (b).

We hereby claim foreign priority benefits under Title 35 United States Code § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

**PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. § 119:**

COUNTRY (if PCT indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. § 119
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

<b>Declaration for Patent Application (Continued)</b> (Includes Reference to PCT International Applications)				ATTORNEY'S DOCKET NUMBER 220002058901	
We hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:					
<b>PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. § 120:</b>					
U.S. APPLICATIONS			STATUS (Check one)		
U S APPLICATION NUMBER	U S FILING DATE	PATENTED	PENDING	ABANDONED	
60/086,232	November 10, 1997			X	
PCT APPLICATIONS DESIGNATING THE U.S.			STATUS (Check one)		
PCT APPLICATION NUMBER	PCT FILING DATE	U S SERIAL NUMBERS ASSIGNED (if any)	PATENTED	PENDING	ABANDONED
PCT/US98/23496	November 4, 1998				X
<b>Send correspondence to:</b> Catherine M. Polizzi Morrison & Foerster LLP 755 Page Mill Road Palo Alto, California 94304-1018			<b>Direct telephone calls to:</b> Catherine M. Polizzi at (650) 813-5651		
201	FULL NAME OF INVENTOR INSEL	FAMILY NAME INSEL	FIRST GIVEN NAME Paul	SECOND GIVEN NAME A.	
	RESIDENCE & CITIZENSHIP La Jolla	CITY La Jolla	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP United States	
	POST OFFICE ADDRESS 8660 Kilbourn Drive	POST OFFICE ADDRESS 8660 Kilbourn Drive	CITY La Jolla	STATE & ZIP CODE/COUNTRY California 92037	
202	FULL NAME OF INVENTOR HERRMANN	FAMILY NAME HERRMANN	FIRST GIVEN NAME Volker	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP Freiburg	CITY Freiburg	STATE OR FOREIGN COUNTRY Germany	COUNTRY OF CITIZENSHIP Germany	
	POST OFFICE ADDRESS In der Roete 18	POST OFFICE ADDRESS In der Roete 18	CITY 79104 Freiburg	STATE & ZIP CODE/COUNTRY Germany	
203	FULL NAME OF INVENTOR BUESCHER	FAMILY NAME BUESCHER	FIRST GIVEN NAME Rainer	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP Essen	CITY Essen	STATE OR FOREIGN COUNTRY Germany	COUNTRY OF CITIZENSHIP Germany	
	POST OFFICE ADDRESS Germarkenstraße 84	POST OFFICE ADDRESS Germarkenstraße 84	CITY 45147 Essen	STATE & ZIP CODE/COUNTRY Germany	
204	FULL NAME OF INVENTOR LIGGETT	FAMILY NAME LIGGETT	FIRST GIVEN NAME Stephen	SECOND GIVEN NAME B.	
	RESIDENCE & CITIZENSHIP Cincinnati	CITY Cincinnati	STATE OR FOREIGN COUNTRY Ohio	COUNTRY OF CITIZENSHIP United States	
	POST OFFICE ADDRESS 8020 Elbrecht Drive	POST OFFICE ADDRESS 8020 Elbrecht Drive	CITY Cincinnati	STATE & ZIP CODE/COUNTRY Ohio 45242	
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.					
SIGNATURE OF INVENTOR 201		SIGNATURE OF INVENTOR 202		SIGNATURE OF INVENTOR 203	
DATE		DATE		DATE 10/13/00	
SIGNATURE OF INVENTOR 204					
DATE					

**DECLARATION FOR PATENT APPLICATION**  
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER  
220002058901

As a below named inventors we hereby declare that:

Our residence, post office address and citizenship are as stated below next to our name,

We believe we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**METHODS AND COMPOSITIONS FOR IDENTIFYING VARIATIONS IN HUMAN  $\alpha_{1B}$  AND  $\beta_2$ -ADRENERGIC RECEPTOR GENES**

the specification of which (check only one item below):

- ☐ is attached hereto.
- ☒ was filed as United States application  
Serial No. 09/402,224  
on September 29, 1999  
and was amended on (if applicable).
- ☐ was filed as PCT international application  
Number  
on  
and was amended under PCT Article 19  
on (if applicable).

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37 Code of Federal Regulations § 1.56(a) and (b).

We hereby claim foreign priority benefits under Title 35 United States Code § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

**PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. § 119:**

COUNTRY (if PCT indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. § 119
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

<b>Declaration for Patent Application (Continued)</b> (Includes Reference to PCT International Applications)				ATTORNEY'S DOCKET NUMBER 220002058901	
We hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:					
<b>PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. § 120:</b>					
U.S. APPLICATIONS			STATUS (Check one)		
U S APPLICATION NUMBER	U S FILING DATE	PATENTED	PENDING	ABANDONED	
60/086,232	November 10, 1997			X	
PCT APPLICATIONS DESIGNATING THE U.S.			STATUS (Check one)		
PCT APPLICATION NUMBER	PCT FILING DATE	U S SERIAL NUMBERS ASSIGNED (if any)	PATENTED	PENDING	ABANDONED
PCT/US98/23496	November 4, 1998				X
Send correspondence to: Catherine M. Polizzi Morrison & Foerster LLP 755 Page Mill Road Palo Alto, California 94304-1018			Direct telephone calls to: Catherine M. Polizzi at (650) 813-5651		
201	FULL NAME OF INVENTOR	FAMILY NAME INSEL	FIRST GIVEN NAME Paul	SECOND GIVEN NAME A.	
	RESIDENCE & CITIZENSHIP	CITY La Jolla	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP United States	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS 8660 Kilbourn Drive	CITY La Jolla	STATE & ZIP CODE/COUNTRY California 92037	
202	FULL NAME OF INVENTOR	FAMILY NAME HERRMANN	FIRST GIVEN NAME Volker	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP	CITY Freiburg	STATE OR FOREIGN COUNTRY Germany	COUNTRY OF CITIZENSHIP Germany	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS In der Roete 18	CITY 79104 Freiburg	STATE & ZIP CODE/COUNTRY Germany	
203	FULL NAME OF INVENTOR	FAMILY NAME BUESCHER	FIRST GIVEN NAME Rainer	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP	CITY Essen	STATE OR FOREIGN COUNTRY Germany	COUNTRY OF CITIZENSHIP Germany	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS Germarkenstraße 84	CITY 45147 Essen	STATE & ZIP CODE/COUNTRY Germany	
204	FULL NAME OF INVENTOR	FAMILY NAME LIGGETT	FIRST GIVEN NAME Stephen	SECOND GIVEN NAME B.	
	RESIDENCE & CITIZENSHIP	CITY Cincinnati	STATE OR FOREIGN COUNTRY Ohio	COUNTRY OF CITIZENSHIP United States	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS 8020 Elbrecht Drive	CITY Cincinnati	STATE & ZIP CODE/COUNTRY Ohio 45242	
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.					
SIGNATURE OF INVENTOR 201		SIGNATURE OF INVENTOR 202		SIGNATURE OF INVENTOR 203	
DATE		DATE		DATE	
SIGNATURE OF INVENTOR 201		SIGNATURE OF INVENTOR 202		SIGNATURE OF INVENTOR 203	
DATE 10/31/00		DATE		DATE	